With Compliments of
CIBA Pharma Private Ltd
BOMBAY.

The Popular Book Depot (Regd)
Lamington Road, Bombay-7

CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol 12 Hormone Production in Endocrine Tumours

A leaflet giving fuller details of available earlier volumes in this series and also the Ciba Foundation General Symposia and Colloquia on Ageing is available from the Publishers

CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME 12

Hormone Production in Endocrine Tumours

Editors for the Ciba Foundation

G E W WOLSTENHOLME,
O.B.E M.A. M B B Ch

and

MAEVE O CONNOR
B A

With 58 Illustrations
and
Cumulative Index to Volumes 1–12



LONDON
J & A CHURCHILL LTD
104 GLOUCESTER PLACE W 1
1958

THE CIBA FOUNDATION

for the Promotion of International Co operation in Medical and Chemical Research

41 PORTLAND PLACE LONDON W 1

Printed in Great Britain

PREFACE

This is the fortieth volume of proceedings of the Ciba Foundation s small informal international conferences and the twelfth in the series of Colloquia on Endocrinology The colloquium reported here was the eighteenth to be concerned with hormonal research five previous volumes in the Endo crinology series each contained the proceedings of two colloquia whilst one discussion on Steroid Nomenclature was published elsewhere in the form of a report The opportunity has been taken to include in this book an index to these first twelve volumes

Dr Ralph Dorfman who proved a most stimulating Chair man had also contributed expertly to the organization of this colloquium a debt which the Director of the Foundation happily acknowledges whilst retaining full responsibility for any defects and deficiencies

The facilities of the Ciba Foundation and the judgment of its officers limited membership as usual to a small group These published proceedings are intended however for a world wide readership especially in regions where recent and adequately representative literature is difficult to come by It is believed that the book brings together material of value to many endocrinologists biochemists, and cancer research workers wherever they may be

Some readers may not be aware that the Ciba Foundation is an educational and scientific Charity administered by its distinguished Trustees and Members of Council in entire independence of the CIBA firm which from its world wide interests provides the Foundation s financial support

The Foundation occupies a house nearly 200 years old in the medical heart of London where accommodation is provided each year for nearly 1 000 scientists from thirty to forty different countries Its activities include conferences such as

that reported here many briefer meetings, annual lectureships, a postgraduate exchange scheme between France and Great Britain, special support of basic research on the problems of ageing a library service in special fields, and as much assistance as can be managed to international organizations, scientific societies and individual research workers

CONTENTS

List of those participating in or attending the Colloquium on Hormone Production in Endocrine Tumours 24th–26th June 1957

R E Bolinger	Second Medical Service Wilhelmina Gasthuis Amsterdam and University of Kansas
E Boyland	Chester Beatty Research Institute London
A C Crooke	Dept of Clinical Endocrinology The Birmingham and Midland Hospital for Women Birmingham
R I Dorfman	Worcester Foundation for Experimental Biology Shrewsbury Massachusetts
U S von Euler	Karolinska Institute Stockholm
M Finkelstein	Hebrew University Hadassah Medical School Jerusalem
J Furth	Dept of Pathology Children s Cancer Research Foundation Boston Massachusetts
W U Gardner	Dept of Anatomy Yale University School of Medicine New Haven Connecticut
C H Gray	Dept of Chemical Pathology King s College Hospital London
J Groen	Second Medical Service Wilhelmina Gasthuis Amsterdam
C Hamburger	Hormone Dept Statens Seruminstitut Copenhagen
E S Horning	Chester Beatty Research Institute London
R A. Huseby	University of Colorado Medical Center Denver
V R Khanolkar	Indian Cancer Research Center Bombay
J H Leathem	Bureau of Biological Research Rutgers University New Brunswick
R Luft	Endocrine Dept Serafimerlasarettet Stockholm
O Mühlbock	Netherlands Cancer Institute Amsterdam
V J O Donnell	University Dept of Steroid Biochemistry Royal Infirmary Glasgow
A S Parkes	National Institute for Medical Research London
G Pincus	Worcester Foundation for Experimental Biology Shrewsbury Massachusetts

J R K PREEDY	Medical Unit London Hospital London
ALICE M ROBINSON	Cancer Research Unit St Bartholomew's Hospital London
E F SCOWEN	St Bartholomew's Hospital London
T SYMINGTON	University Dept of Pathology Royal Infirmary Glasgow
J R TATA	National Institute for Medical Research London and India
J VALLANCE OWEN	Postgraduate Medical School of London
A F WILLEBRANDS	Second Medical Service Wilhelmina Gasthuis Amsterdam
G WOOLLEY	Sloan Kettering Institute for Cancer Research New York
J M YOFFEY	Dept of Anatomy University of Bristol

CHAIRMAN'S OPENING REMARKS

R I Dorfman

This meeting is concerned with hormone producing tumours the functional tumours of the endocrine system and we might say that the active work in this field has been in progress for approximately 20 to 25 years During this period studies in this field have centred particularly around aetiology physiology and pathology Until recently few biochemical were reported A great change has occurred It is now possible to study these tumours at a more intimate level the biochemical level This is so because new micromethods are available for the bioassay and chemical characterization of the hormonal materials Specifically we have available precision chromatography radioactive tracers and highly refined techniques in the field of spectroscopy so much so that microgram of material can actually be isolated in almost the full of the word True we cannot take melting points and recrystallize these microgram quantities in the classical ways but in place of these older methods we have new and elegant tricks These new micromethods give us the means to find biochemical facts about the functional tumours and permit us to correlate this information with the more classical techniques that have been used previously During this meeting we shall hear a good deal about these new methods and these new correlations

We might also focus our attention momentarily on a few questions that might be profitably kept in mind during the of this meeting Some of these questions are at the mental level and will be discussed I believe in real detail and as a result stimulate further thought and intensify effort in this field I have jotted down a few at random which have been of interest at least to me and may be

suggestive to you One that has recurred through the literature for some time now is concerned with the question of whether tumours actually contain special or newly developed or unique biosynthetic enzyme systems for the synthesis of these active materials This is still an open question Do functional tumours produce compounds which are unique and characteristic for tumours as compared to those materials produced by the corresponding normal tissues? If this question could be answered in the affirmative we obviously would have an important approach to a diagnostic test Thus far studies in this area have indicated that the products are not unique A second question pertains to whether a general hypothesis can be enunciated to account for the induction of functional tumours Some members of this group have given a considerable amount of thought to this problem and new ideas may be forthcoming A third question that will I am *sure, be brought before us will concern the question of autonomy* and dependence of functional tumours In greater detail, we may ask when and how a tumour becomes independent Along these lines we also have the question of whether tumours can be both malignant and dependent Recent work on this point has raised some questions of both mechanism and definitions Finally, we may ask just how valuable the newer biochemical techniques really are in advancing our knowledge of functional tumours

Many more questions could be asked but I am sure that at this point you are more anxious to hear the answers I shall only say at this time *that I am honoured and delighted to be* your Chairman

EXPERIMENTAL PITUITARY TUMOURS*

JACOB FURTH AND KELLY H CLIFTON

The Children s Cancer Research Foundation Children s Medical Center and Department of Pathology Harvard Medical School Boston

THE pituitary gland is the centre of a marvellous automation regulating a vast number of body functions This is accomplished by a mosaic of cell types independently operating emitting regulatory substances to their target systems and receiving impulses either from their target systems or from higher centres stimulated through circuitous routes hitherto not fully explored

The work to be presented is the fragmentary beginning of attempts at isolation of those functional units of the pituitary gland called the tropic cells The research to be reviewed is limited to consideration of three independent systems extensively studied (Furth, 1955 Furth and Clifton 1957) and to a fourth which is still poorly understood The fact that the different tropic cells are situated side by side in a mosaic suggests the possibility that they may receive and transmit information to each other but this area of thought has not yet been explored Much is known about the hypothalamus playing a role in feed back regulation of some functions of pituitary tropic cells but in part for simplicity of presentation of the pituitary mosaic in part because of lack of first hand knowledge it will not be considered here

Nomenclature The tropic cells of the pituitary will be designated after their main target organ by the suffix trope as thyrotrope adrenotrope mammotrope somatotrope, and gonadotrope (originally proposed by Purves and Griesbach) and abbreviated Tt At Mt St etc respectively Addition

Work supported by Grant C-2259 of National Institutes of Health and by the United States Atomic Energy Commission

of H" to the symbols of tropic cells will designate their hormones (TtH, AtH, MtH StH, etc) As a synonym for the hormones the use of the suffix "tropin" will be accepted Thus, the hormones will also be spoken of as thyrotropin, adrenotropin mammotropin, etc

Survey Table I surveys the monomorphous tumours investigated and their salient hormonal effects Best studied

Table I
HORMONAL EFFECTS

Tumour	*TtH*	*GtH*	*bte*	*AtH*	*m.s*	*MtH*	*StH*
TtT	+++	+	+			0	+*
AtT	0	0	0	+++	+++	0**	0
MtT	0	0	0	0		+++	++
?StT	+	0	0	0		++	+++

bte = biliary tract ectasia
m s = melanocyte stimulation
indirect by way of thyroid hormone
enhancement of activity notably secretion

among them are the thyrotropes which have an apparently built in gonadotropin effect in a ratio guessed at somewhere near 1 1000 that is whenever the thyrotropic activity is high there is an accompanying gonadotropic activity A remarkable and constant secondary change biliary tract ectasia specific to thyrotropes will be discussed The adrenotropes possess only adrenal gland stimulating activity in their natural hosts Their melanocyte stimulating activity is inherent in their chemical structure The mammotropes have a built in somatotropic effect The possible existence of another somatotrope will be discussed A slight somatotropic effect of thyrotropes is exerted by way of thyroid hormone and is not a specific property of TtH

These tropic tumour cells will now be discussed more fully Because of the large amount of material to be covered our survey will be sketchy and pictorial, leaving clarification of inadequately presented topics for the discussion

Thyrotropic tumours Thyrotropic pituitary tumours can be induced by sustained thyroid deficiency brought about by any one of four procedures radiothyroidectomy (Gorbman 1949) surgical thyroidectomy (Dent, Gadsden and Furth 1955) antithyroidal compounds (Moore Brackney and Bock 1953) and low iodine diet (Axelrad and Leblond 1955) This is accomplished by interference with the physiological feed back mechanism and sustained stimulation of thyrotropes This sequence of events appears to be a must following thyroidectomy in every strain of mice and in every animal It can however be interrupted by the administration of thyroid hormone (see Dent Gadsden and Furth 1955) Primary thyrotropic tumours could be transplanted only to animals with marked thyroid hormonal deficiency but not to normals Rarely thyrotropic tumours are encountered spontaneously (Bielschowsky 1953 Furth and Buffett 1957 unpublished data) and among animals that had been exposed to X rays (Furth and Buffett 1957 unpublished data) All of those thus far tested were autonomous at the start

Dependent grafted thyrotropic tumours attain large size weighing as much as 10 g and metastasize to regional nodes only They are a rich source of thyroid hormone The hormonal content varies with the strain passage and character of the tumour Soon after isolation the hormonal content of some dependent tumours was as much as ten times that of powders of sheep and cattle pituitary (The normal rodent pituitary contains 0 1-0 2 U S P units per mg of dry weight) After successive passages the hormonal secretion of TtT dropped gradually With two transplantable strains extensively studied by Bates Anderson and Furth (1957) it appeared to be stabilized at about the level of the normal pituitary but fell rapidly with a third strain to about one tenth of the original potency

of 'H to the symbols of tropic cells will designate their hormones (TtH, AtH MtH StH, etc) As a synonym for the hormones, the use of the suffix 'tropin' will be accepted Thus the hormones will also be spoken of as thyrotropin adrenotropin mammotropin etc

Survey Table I surveys the monomorphous tumours investigated and their salient hormonal effects Best studied

Table I

HORMONAL EFFECTS

Tumour	*TtH*	*GtH*	*bte*	*AtH*	*m s*	*MtH*	*StH*
TtT	+++	+	+			0	+*
AtT	0	0	0	+++	+++	0**	0
MtT	0	0	0	0		+++	++
?StT	+	0	0	0		++	+++

bte = biliary tract ectasia
m s = melanocyte stimulation
* indirect by way of thyroid hormone
** enhancement of activity notably secretion

among them are the thyrotropes which have an apparently built in gonadotropin effect in a ratio guessed at somewhere near 1 1000 that is whenever the thyrotropic activity is high there is an accompanying gonadotropic activity A remarkable and constant secondary change biliary tract ectasia specific to thyrotropes will be discussed The adrenotropes possess only adrenal gland stimulating activity in their natural hosts Their melanocyte stimulating activity is inherent in their chemical structure The mammotropes have a built in somatotropic effect The possible existence of another somatotrope will be discussed A slight somatotropic effect of thyrotropes is exerted by way of thyroid hormone and is not a specific property of TtH

These tropic tumour cells will now be discussed more fully Because of the large amount of material to be covered, our survey will be sketchy and pictorial, leaving clarification of inadequately presented topics for the discussion

Thyrotropic tumours Thyrotropic pituitary tumours can be induced by sustained thyroid deficiency brought about by any one of four procedures radiothyroidectomy (Gorbman, 1949) surgical thyroidectomy (Dent, Gadsden and Furth 1955) antithyroidal compounds (Moore Brackney and Bock 1953) and low iodine diet (Axelrad and Leblond 1955) This is accomplished by interference with the physiological feed back mechanism and sustained stimulation of thyrotropes This sequence of events appears to be a must following thyroidectomy in every strain of mice and in every animal It can however be interrupted by the administration of thyroid hormone (see Dent Gadsden and Furth 1955) Primary thyrotropic tumours could be transplanted only to animals with marked thyroid hormonal deficiency but not to normals Rarely thyrotropic tumours are encountered spontaneously (Bielschowsky 1953, Furth and Buffett 1957 unpublished data) and among animals that had been exposed to X rays (Furth and Buffett 1957, unpublished data) All of those thus far tested were autonomous at the start

Dependent grafted thyrotropic tumours attain large size weighing as much as 10 g and metastasize to regional nodes only They are a rich source of thyroid hormone The hormonal content varies with the strain passage and character of the tumour Soon after isolation the hormonal content of some dependent tumours was as much as ten times that of powders of sheep and cattle pituitary (The normal rodent pituitary contains 0 1–0 2 U S P units per mg of dry weight) After successive passages the hormonal secretion of TtT dropped gradually With two transplantable strains extensively studied by Bates Anderson and Furth (1957) it appeared to be stabilized at about the level of the normal pituitary but fell rapidly with a third strain to about one tenth of the original potency

The concentration of thyrotropins in the blood serum of mice with large tumours was about 1 unit per ml, or approximately 2,000 times the normal level Whereas in the normal man the amount of thyrotropins in the blood is approximately 30 per cent of that of the pituitary gland, in tumour bearing mice the blood concentration is only about 1 per cent of its source i e the tumour (Bates, Anderson and Furth 1957) Thus although thyrotropes release hormone in extracranial location it is possible that lack of connexion with hypothalamic centres retards release of the hormone

With acquisition of autonomy the thyrotropin concentration dropped and with a few highly autonomous tumours it ceased entirely as indicated by changes in tumour bearing hosts *After several generations of successive passages* almost all transplanted thyrotropic tumours turned autonomous However, the fully dependent state, unlike that of most endocrine tumours lasts for several years making this neoplasm an excellent material for the study of stages of transformation from dependency to full autonomy When autonomy is attained and the tumour is grafted on normal mice the thyroid gland of the tumour bearing host is an excellent indicator of the quantity of thyrotropin secreted With *highly functional tumours it becomes tremendously enlarged* Adenomatoid nodules develop which sometimes metastasize to regional lymph nodes and invade blood vessels Nevertheless these metastasizing thyroid adenomas are fully dependent neoplasms in that they cannot be transplanted to normal hosts

The autonomous thyrotropic tumours which were isolated from normal or radiothyroidectomized animals were hormone responsive They grew very slowly in normal hosts Their growth could be greatly enhanced by the administration of propylthiouracil *Cessation of conditioning with propylthiouracil* markedly reduced their growth rate

The following is a hint to those working with functional tumours It is desirable to preserve them in highly functional state soon after isolation by freezing at low temperature (below −60°C) When in the course of animal passages the

tumour loses its functional capacity the original tumour can be resuscitated from the frozen state in a highly active form as was done by us repeatedly

The thyrotropes appear to have a built in gonadotropic effect Whenever the thyroid hormonal level is high, there is a marked ovarian stimulation resembling that seen in normal mice receiving quantities of gonadotropins There is follicular maturation with haemorrhage and secondary stimulation of the uterine horn When this change is extensive there is some hyperplasia of the mammary gland as seen in mice receiving oestrogenic hormone Hyperplasia of the mammary gland we believe is due to secondary stimulation of mammo tropes by the oestrogens secreted by the stimulated ovaries This is a remarkable example of Claude Bernard s determin ism of how one event may cause a secondary and this a tertiary event and so on proceeding throughout the entire life span of the host and terminating in death

Purification studies of Bates (personal communication) suggest that the gonadotropin effect is due to the presence of a gonadotropin in trace quantities rather than to a side effect of a large thyrotropin molecule

Another remarkable secondary change is a cystic biliary tract ectasia (Furth Gadsden and Upton 1952) a truly puzzling phenomenon Such a change was noted earlier by Gardner Allen and Smith (1941) in mice receiving large doses of oestrogen but it cannot be directly attributed to oestrogens since it occurred with equal frequency in mice of both sexes The ampulla and the ducts were patent hyperplastic and only secondarily inflamed Consequently the change may be related to some hormonal mechanism responsible for emptying the extrahepatic biliary tract The change is most marked in animals with dependent tumours and its degree appears to be directly related to the duration of the tumour bearing period and the level of hormone secreted It is present in athyroid as well as in hyperthyroid hosts bearing highly secretory autonomous tumours Thus it is not related to thyroid hormone which recirculates through the biliary tree

and intestinal tract It is conceivably related to binding or exhaustion of the hormone inactivating mechanism of the liver and secretion of abnormal products into the biliary tract Biliary tract ectasia and the gonadotropic effect just de scribed were never seen with mammotropic, adrenotropic, and somatotropic tumours

The weight changes in thyroidectomized mice bearing moderately functional dependent thyrotropic pituitary tumours were analysed by Bates, Anderson and Furth (1957) The slight increase in body weight of thyroidectomized mice is roughly related to tumour weight However there is a distinct increase in body weight and organ weights in intact animals bearing highly secreting autonomous tumours due in all probability to sustained thyroxine excess The weights of the adrenals the male gonads and the seminal vesicles are approximately normal the ovary and uterus, on the contrary are greatly enlarged as has already been discussed Pituitary hyperplasia was according to these data, less extensive in thyroidectomized hosts of thyrotropic tumours than in those without tumours but formation of primary pituitary growth was not prevented

Known histochemical techniques, such as the trichrome stains the periodic Schiff reaction and the aldehyde fuchsin stain failed in our hands, to identify highly secreting tumorous thyrotropes even though their probable ancestral cell had given these reactions (Halmi and Gude 1954) On the basis of known histochemical reaction these thyrotropes and the adrenotropes to be described would be classified as chromo phobes This study of pituitary tumours led us to conclude that the chromophobe is not a separate non secretory cell type Some 20 chromophobic tumours occurring in normal or irradiated animals were assayed in transplantation studies in addition to those induced by thyroidectomy and all proved to be secretory although the level of secretion was sometimes very low We assume that non responsiveness of the target organs of the original host is, in many instances directly related to the genesis of the tumour



FIG 1 Electron micrograph of a thyrotropic tumour showing

A mass of lipid (?)
B ergastoplasm
C mitochondria
D erythrocyte
E endothelial cell
G secretory ovoid granules

[facing page 8

and intestinal tract It is conceivably related to binding or exhaustion of the hormone inactivating mechanism of the liver and secretion of abnormal products into the biliary tract Biliary tract ectasia, and the gonadotropic effect just described, were never seen with mammotropic adrenotropic and somatotropic tumours

The weight changes in thyroidectomized mice bearing moderately functional dependent thyrotropic pituitary tumours were analysed by Bates Anderson and Furth (1957) The slight increase in body weight of thyroidectomized mice is roughly related to tumour weight However there is a distinct increase in body weight and organ weights in intact animals bearing highly secreting autonomous tumours due in all probability, to sustained thyroxine excess The weights of the adrenals the male gonads and the seminal vesicles are approximately normal the ovary and uterus, on the contrary are greatly enlarged, as has already been discussed Pituitary hyperplasia was according to these data, less extensive in *thyroidectomized hosts of thyrotropic tumours than in those* without tumours but formation of primary pituitary growth was not prevented

Known histochemical techniques such as the trichrome stains the periodic Schiff reaction and the aldehyde fuchsin stain failed in our hands, to identify highly secreting tumorous thyrotropes even though their probable ancestral cell had given these reactions (Halmi and Gude 1954) On the basis of known histochemical reaction these thyrotropes and the adrenotropes to be described would be classified as chromophobes This study of pituitary tumours led us to conclude that the chromophobe is not a separate non secretory cell type Some 20 chromophobic tumours occurring in normal or irradiated animals were assayed in transplantation studies in addition to those induced by thyroidectomy and all proved to be secretory, although the level of secretion was sometimes very low We assume that non responsiveness of the target organs of the original host is in many instances directly related to the genesis of the tumour



FIG 1 Electron micrograph of a thyrotropic tumour showing

A mass of lipid (?)
B ergastoplasm
C mitochondria
D erythrocyte
E endothelial cell
G secretory ovoid granules

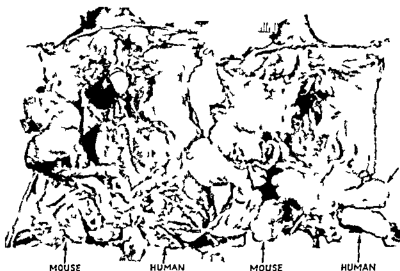


FIG 2 Mice bearing a grafted adrenotropic mouse tumour in the right thigh and a human tumour in the left thigh Arrows point to tumours

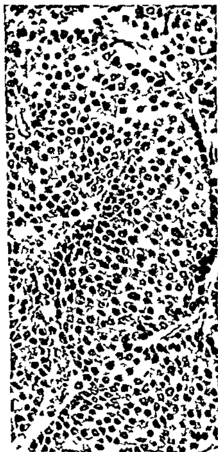


Fig 3 (a)

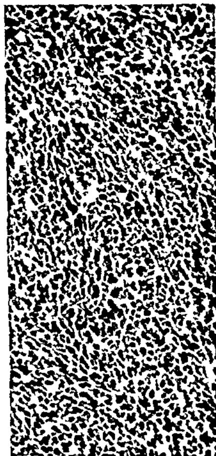


Fig 3 (b)

Fig 3 Microscopic picture (× 340) of
(a) the adrenotropic mouse tumour and
(b) the human tumour from one of the mice shown in Fig 2



Fig 4 Rat bearing a four day old tissue culture of a mammotropic tumour Note the tremendous enlargement of the mammary gland

Electron microscopic studies of Farquhar and Rinehart (1954) indicate distinct differences among the various tropic pituitary cells The electron microscopic picture of functional thyrotropic tumour cells shown in Fig 1 taken by Dr Betty Geren Uzman was interpreted by Dr Marilyn Farquhar The cells contain numerous secretory ovoid granules (marked G) occasional granules are surrounded by a membrane and appear to be arising in a sac like structure The mitochondria appear to be more pleomorphic and the canalicular structure of the endoplasmic reticulum or ergastoplasm more abundant than seen in normal thyrotropes Since the secretory granules of the tropic cells are best conceived as zymogen granules contained in ultramicroscopic structures and probably synthesized by ultramicroscopic organelles it is conceivable that even *non secreting* cells will be identified by the *character of this* ultramicroscopic organelle

Adrenotropic tumours All adrenotropic tumour strains originated in mice exposed to ionizing irradiation Several such strains have been studied in serial passages All are essentially alike All are autonomous differing from each other merely in the degree of hormonal production as indicated by the degree of secondary changes produced by them The tumour cells are small finely granular chromophobic and cannot be distinguished from other non eosinophilic tumour cells Fine granularity of the cytoplasm suggests the presence of secretory granules which should be visible under the electron microscope The tumours seldom metastasize beyond the regional lymph nodes and the cell population exhibits no high degree of autonomy Adrenalectomy appears to increase somewhat the growth rate of the tumours

The changes noted in tumour bearing hosts are in general similar to those noted in animals that have been given purified α corticotropin by Li and co workers (1957) These include stimulation of the adrenal gland in normal and hypophysectomized animals production of marked lymphopaenia with involution of the thymus eosinopaenia and enhancement of fat synthesis Androgenic effects as described by Li and

co workers (1957), have not been noted by us The clinical syndrome of these adrenotropic tumours differs somewhat from Cushing's syndrome but corresponds to known effects of adrenal corticoids of rodents, and the findings indicate that the stimulated adrenals have both gluco and mineralo corticoid activity The occurrence of ovarian atrophy and vaginal mucification (Bahn *et al* 1957*a*, *b*) indicates inhibition of pituitary gonadotropins by the adrenal corticoids secreted as was found by Greep and Jones (1950)

The first change noted in tumour bearing animals is that of an increase in body weight due entirely to adiposity Studies of Mayer Zomzely and Furth (1956) and of Shull and co workers (1956) with adrenotropic tumours indicate that adrenotropins are instrumental in homeostasis of the liver glycogen and of blood glucose The liver glycogen of fasted mice bearing such tumours is several times higher than that of normal fasted siblings, and their blood sugar hardly decreases in comparison there is about a 50 per cent decrease in their normal fasted siblings

The liver glucose 6 phosphatase activity of tumour bearing mice is elevated to about twice that of their siblings By contrast the activity of other enzymes studied such as liver phosphorylase and hexokinase is approximately *unchanged*

Lipogenesis is considerably enhanced (Mayer, Zomzely and Furth 1956) Mice bearing adrenotropic tumours synthesize more fatty acids from labelled acetate not only in the normally fed state but also in the fasted state Increase of cholesterol synthesis from labelled acetate is indicated by elevated blood tissue cholesterol levels This increase in lipogenesis is concomitant with a negative nitrogen balance resulting in replacement of protein by fat in many animals so that even those which do not weigh more than their normal siblings contain two or more times as much body fat

White blood counts show a marked drop to 1000–2000 even before the tumours become palpable This is due primarily to an almost complete *disappearance of lymphocytes Eosinophils*

are usually absent Parallel with leucopaenia lymphopaenia and eosinopaenia is a very marked increase in water intake and urine output The urine output with highly functional tumours exceeds tenfold that of normal The specific gravity of the urine is decreased and rarely there is a marked glycosuria (Bahn *et al* 1957*a* *b*) The polyuric syndrome has been reproduced by Heilman and Kendall (1956) by administration of corticosterone the chief steroid secreted by the adrenal cortex of rats and mice All these changes can be cancelled out almost overnight by adrenalectomy

A characteristic and specific feature of these tumour hosts is an extraordinary sensitivity to infection With highly functional tumours death ensues by generalized infection usually with a common diphtheroid organism which is non pathogenic to normal mice but is fatal to cortisonized animals Therefore it is necessary to carry these tumours in adrenalectomized animals Normal hosts usually die of this infection when the tumour weighs only a few hundred milligrams In adrenalectomized siblings the tumours continue to grow, reaching a weight of several grams without any apparent harm to their hosts

Related to sensitivity to infections is enhancement of tolerance to heterografts Fig 2 shows two animals each of which has an adrenotropic mouse tumour in the right thigh and a human tumour in the left thigh The human tumours usually attain a larger size than the isologous tumours The microscopic appearance of the two tumours in one of these animals is shown in Fig 3

The steroids secreted by the stimulated adrenals of animals bearing AtT were studied by Bahn and co workers (Bahn *et al* 1956 Wilson Borris and Bahn 1958) Extracts of hydrolysed pooled urine were chromatographed on paper in the Bush Zaffaroni system Tumour bearing mice excreted 5 μg of total neutral C_{21} steroids per day of which corticosterone comprised 0 3 μg Only traces of cortisone cortisol and other highly active polar steroids were present No aldosterone activity was demonstrated The excretion of C_{19} ketosteroids

was approximately 34 μg per day of which $C_{19}O_3$ compounds comprised 23 μg

The gonads of tumour bearing animals were atrophic the uteri were approximately normal, and the vaginal mucosa was never completely cornified A slight mammary gland hyper plasia was encountered in a few female mice but this can be attributed to the mammotrope enhancing activity of corti coids not to direct mammotropic action of adrenotropes *per se*

Steelman and co workers (1956) assayed moderately autonomous adrenotropic tumours by the Sayers ascorbic acid depletion technique for AtH activity, and found approxi mately 1 m u of AtH per mg of tumour Cohen Bloch and Celozzi (1957), using an assay based on stimulation of adrenal slices incubated with AtH *in vitro* estimated that fresh tumour contains about 10 times that much AtH This raises the question of the relative merits of the ascorbic acid depletion and *in vitro* synthesis assays The latter is certainly more sensitive and is presumably more specific The high level of melanocyte stimulating activity of tumour extracts was dis covered by Steelman and co workers (1956) Bahn and co workers (1957*a*) noticed that this activity was greatly en hanced by heating at acid pH This is in line with current ideas of the relation between adrenotropin and the melano cyte stimulating hormone of the anterior pituitary the latter being part of the long chain polypeptide adrenotropin molecule (see Li *et al* 1957)

Mammotropic tumours Only a résumé of the salient findings will be given here The mammotropes are character ized by the presence of coarse acidophilic granules in the cytoplasm Mammotropic tumours were observed by us first among mice exposed to whole body ionizing radiation In more recent studies we noted their occurrence among unirradiated very old normal animals (Furth and Buffett 1957, unpublished data) Irradiation certainly hastens their occurrence and possibly increases their frequency All radia tion induced mammotropic tumours were autonomous Several strains were studied in numerous consecutive passages

Ovariectomy in mice will prevent the development of mammotropic tumours Having recognized the relation of mammotropic tumours in the ovary we turned to the study of the oestrogen induced pituitary tumours discovered over 20 years ago by several investigators All of those studied turned out to be mammotropic and acidophilic possessing properties similar to those of radiation induced mammotropic tumours in mice The first generation grafts of oestrogen induced mammotropic tumours proved to be dependent (i e, they grew only in oestrogen treated hosts) but upon successive passages they rapidly acquired autonomy (Furth *et al* 1956) The findings to be detailed were made with strain 4 which is autonomous but still responsive to the physiological stimulants and inhibitors of mammotropes Fig 4 shows a rat bearing a grafted mammotropic tumour in the right thigh Note the profound mammary gland stimulation and the increase in body size and in the size of several organs Somatotropic effect is proportional to mammotropic activity Since the mammotropic tumours appear to be monomorphous they either secrete two hormones, or the native hormone has both somatotropic and mammotropic activities Oestrogen is recognized by us as a specific stimulant of the mammotrope exerting its action on the mammary gland through stimulation of mammotropes There is a direct relation between the dose of oestrogen administered and promotion of the growth of mammotropes (Clifton and Furth 1957) The threshold dose of oestrogen which would stimulate mammotropes is in the neighbourhood of 10 μg of stilboestrol (3 4 di *p* hydroxy phenylhex 3 ene) administered in a single pellet There is a parallelism between the growth of mammotropes and the somatotropic changes

The hormonal secretions of mammotropes were assayed by Bates Clifton and Anderson (1956) using the pigeon crop gland method They found that transplanted rat mammotropic tumours contained only about 10 per cent of the hormone concentration of bovine pituitary Prolactin was not detected in our adrenotropic and thyrotropic tumours Bates is of the

opinion that the secretions of these mammotropic tumours are not identical with prolactin (personal communication) The merits of crop sac assay in detecting mammotropic hormone of mammals are debatable

The availability of transplantable monomorphous pituitary tumours and the ease of their assay by transplantation in homologous hosts led us to attempt to culture them *in vitro* Fig 4 shows the modest beginning of such an attempt Thus far 20 day old tissue cultures have been successfully re implanted in animals causing a tremendous hyperplasia of the mammary gland of the tumour bearing hosts (Furth and Zompetti 1957, unpublished data) With this background knowledge of specific enhancing and retarding influences it may not be too optimistic to hope that it will be possible to grow *in vitro* these monomorphous pituitary tumours with retention of their ability to secrete hormones There is another potential use of these pituitary tumours Inasmuch as they specifically control the function of several of their target organs they might be used for assay of substances inhibiting neoplasms of organs under their control

Complex tumour strains exhibiting predominantly somatotropic activity At a Laurentian Hormone Confer ence (Furth 1955) we described a transplantable pituitary tumour, the predominant feature of which was a marked stimulation of body growth and organ growth Another strain has been isolated recently with a similar spectrum of secondary changes and biological behaviour Increase of body weight with this tumour is not associated with obesity The animals are lean and there is a profound stimulation of protein synthesis The nuclei of liver cells are tremendously enlarged and mitotic figures in liver cells are numerous There is also an increase in cytoplasmic basophilia Although no biosynthetic studies have been made thus far with this tumour it appears to stimulate protein and nucleoprotein synthesis Moderate enlargement of the thyroid is a character istic secondary change with these two tumour strains How ever in contrast to thyrotropic tumours decreasing thyroid

hormone levels of the hosts reduced the growth rate of these tumours instead of enhancing it Occasionally there was in females, a moderate mammary gland hyperplasia, but in contrast to mammotropic tumours administration of oestrogen retarded the growth of the tumour instead of enhancing it The tumour hosts had a moderate polydipsia and polyuria as did the animals bearing adrenotropic tumours However, their adrenals were lipid rich and lack of eosinopaenia lymphopaenia and failure of thymic involution in tumour bearing hosts are ample evidence for lack of hypersecretion of corticoids These complex tumour strains originated in irradiated animals and were highly autonomous

At present we are attempting to analyse these strains along with some 18 other recently isolated pituitary tumour strains by a procedure shown in Table II To test for autonomy the

Table II

SCHEME OF TRANSPLANTATION ANALYSIS

Assay for	*Treatment of host*
Autonomy	None
Hormonal spectrum	Hypophysectomy
Dependency and responsiveness	*Specific stimulation*
Thyroidectomy or propylthiouracil	Tt
Adrenalectomy	At
Oestrogen	Mt
? Gonadectomy	Gt
?	St

tumours are grafted on normal histocompatible hosts Their hormonal spectrum is assayed from time to time in hypophysectomized animals To determine their dependency and responsiveness respectively they are grafted on variously

conditioned hosts, thyroidectomy enhances growth of thyrotropes adrenalectomy that of adrenotropes, oestrogenization of the hosts stimulates mammotropes and gonadectomy is presumed to stimulate gonadotropes Attempts to induce gonadotropic tumours have thus far been unsuccessful and we have no idea what regulates somatotropes and thus how to cheat their feed back mechanism and make them become tumorous

Thus was made an inroad into a dissection of the pituitary into its anatomically functional units It may not appear to be too optimistic to hope that with persistence and some serendipity, monomorphous masses will be obtained from as many types of pituitary cells as there are in this organ

REFERENCES

AXELRAD A A and LEBLOND C P (1955) *Cancer N Y* 8 339
BAHN R ANDERSON E WILSON H and FURTH J (1957a) *Canad J Biochem Physiol* in press
BAHN R FURTH J ANDERSON E and GADSDEN E (1957b) *Amer J Path* 33 1075
BAHN R WILSON H ANDERSON E and FURTH J (1956) XXth *Int physiol Congr* Abstracts p 54
BATES R W ANDERSON E and FURTH J (1957) *Endocrinology* 61 549
BATES R W CLIFTON K H and ANDERSON E (1956) *Proc Soc exp Biol N Y* 93 525
BIELSCHOWSKY F (1953) *Brit J Cancer* 7 203
CLIFTON K H and FURTH J (1957) *Proc Soc exp Biol N Y* 94 809
COHEN A I BLOCH E and CELOZZI E (1957) *Proc Soc exp Biol N Y* 95 304
DENT J N GADSDEN E I and FURTH J (1955) *Cancer Res* 15 70
FARQUHAR M G and RINEHART J F (1954) *Endocrinology* 55 857
FURTH J (1955) *Recent Progr Hormone Res* 11 221
FURTH J and CLIFTON K H (1957) *Cancer N Y* 10 842
FURTH J CLIFTON K H GADSDEN E and BUFFETT R F (1956) *Cancer Res* 16 608
FURTH J GADSDEN E L and UPTON A C (1952) *Cancer Res* 12 739
GARDNER W U ALLEN E and SMITH G M (1941) *Proc Soc exp Biol N Y* 46 511
GORBMAN A (1949) *Proc Soc exp Biol N Y* 71 237

GREEP R O and JONES C (1950) *Recent Progr Hormone Res* 5 197
HALMI N S and GUDE W D (1954) *Amer J Path* 30, 403
HEILMAN F R and KENDALL E C (1956) *Proc Mayo Clin* 31 454
LI C H FØNSS BECH P GESCHWIND I I HAYASHIDA T HUNGERFORD G F LOSTROH A J LYONS W R MOON H D REINHARDT W O and SIDEMAN M (1957) *J exp Med* 105 335
MAYER J ZOMZELY C and FURTH J (1956) *Science* 123 184
MOORE G E BRACKNEY E L and BOCK I G (1953) *Proc Soc exp Biol N Y* 82 643
SHULL K H CAHILL, G GADSDEN E and MAYER J (1956) *J biol Chem* 222 415
STEELMAN S L KELLY T L NORGELLO H and WEBER G F (1956) *Proc Soc exp Biol N Y* 92 392
WILSON H BORRIS J J and BAHN R C (1958) *Endocrinology* in press

DISCUSSION

Dorfman I think you will all agree with me that Dr Furth s work has been an elegant model to illustrate the power of the joint use of classical methods and the newer aspects of biochemistry I must also add that he has excited biochemists to take up studies in this field with profit to the field and themselves

Gardner What percentage of irradiated animals would show adrenotrophic tumours and about how long after irradiation do such tumours appear? Have you been able to isolate polyfunctional tumours in different lines or strains derived from one tumour?

Furth Three of the first ten radiation induced pituitary tumours assayed were adrenotrophic and six mammo somatotrophic The possibility that some primary tumours are polyfunctional or mixed was recognized recently and at present we have 18 tumours isolated from irradiated animals which are being carried in successive passages in normal and variously conditioned hosts All new adrenotrophic tumour strains turned out to be true to type When oestrogen was given to some hosts mammotrophic effects appeared This we believe is due to the ability of glucocorticoids to enhance the effects of mammotropes Thyroid stimulation was not noted in mice bearing adrenotrophic tumours Two thyrotrophic tumours were identified in about 1 500 irradiated mice and one in 200 unirradiated controls

Regarding transformation of types and the possibility of induction of mixed or polyfunctional tumours we shall have to wait for another few years when the current studies will be completed Our present concept is that mixed tumours may occur as they do in the irradiated ovary Every cell can be altered by radiation and the conditions of the host on which the primary tumours are grafted may select or suppress certain cell types Following isolation in normal hosts all adrenotrophic and mammotrophic strains remained fixed and true to type as described

Gardner I never saw an adrenotrophic tumour until about 18 months ago We now have one that is much the same as Dr Furth has described

conditioned hosts, thyroidectomy enhances growth of thyro tropes adrenalectomy that of adrenotropes, oestrogenization of the hosts stimulates mammotropes, and gonadectomy is presumed to stimulate gonadotropes Attempts to induce gonadotropic tumours have thus far been unsuccessful and we have no idea what regulates somatotropes and thus how to cheat their feed back mechanism and make them become tumorous

Thus was made an inroad into a dissection of the pituitary into its anatomically functional units It may not appear to be too optimistic to hope that with persistence and some serendipity monomorphous masses will be obtained from as many types of pituitary cells as there are in this organ

REFERENCES

AXELRAD A A and LEBLOND C P (1955) *Cancer N Y* 8 339

BAHN R ANDERSON E WILSON H and FURTH J (1957a) *Canad J Biochem Physiol* in press

BAHN R FURTH J ANDERSON E and GADSDEN F (1957b) *Amer J Path* 33 1075

BAHN R WILSON H ANDERSON E and FURTH J (1956) XXth *Int physiol Congr* Abstracts p 54

BATES R W ANDERSON E and FURTH J (1957) *Endocrinology* 61 549

BATES R W CLIFTON K H and ANDERSON E (1956) *Proc Soc exp Biol N Y* 93 525

BIELSCHOWSKY F (1953) *Brit J Cancer* 7 203

CLIFTON K H and FURTH J (1957) *Proc Soc exp Biol N Y* 94 809

COHEN A I BLOCH E and CELOZZI E (1957) *Proc Soc exp Biol N Y* 95 304

DENT J N GADSDEN E L and FURTH J (1955) *Cancer Res* 15 70

FARQUHAR M G and RINEHART J F (1954) *Endocrinology* 55 857

FURTH J (1955) *Recent Progr Hormone Res* 11 221

FURTH J and CLIFTON K H (1957) *Cancer N Y* 10 842

FURTH J CLIFTON K H GADSDEN E and BUFFETT R F (1956) *Cancer Res* 16 608

FURTH J GADSDEN E L and UPTON A C (1952) *Cancer Res* 12 739

GARDNER W U ALLEN E and SMITH G M (1941) *Proc Soc exp Biol N Y* 46 511

GORBMAN A (1949) *Proc Soc exp Biol N Y* 71 237

of cortisone So one feels that the response to a tumour producing ACTH may be partly indirect and due to side effects of the increased corticoid secretion on other pituitary hormones

Furth That adrenotrophic tumours act by way of the adrenals is indicated by adrenalectomy promptly abolishing all secondary changes caused by them The sequence of changes to thyrotrope another monomorphous tumour culminates in a polyglandular insufficiency The thyrotropes appear to have a slight built in gonadotrophic (HTS?) potency The enlarged ovary secretes excessive amounts of oestrogen the latter in turn stimulates secretion of mammotrophin and this in turn the mammary gland Now how do we know that this is the sequence? A graft of TSH secreting tumour in a hypophysecto mized animal causes ovarian and uterine stimulation but not mammary gland hyperplasia Removal of the ovaries will abolish the mammary gland stimulation This is an example of how a single trauma radio thyroidectomy sets in motion a fascinating cycle resulting in a multi hormonal disturbance reminding one of Claude Bernard s principle of determinism

Dorfman The stimulation of your tumours by ACTH brings up some interesting points First there appears to be a discrepancy between the ascorbic acid titres of the ACTH and the ability of this material to stimu late hormone formation by the quartered rat adrenal

Furth The rat adrenal corticoid tumour responds to ACTH better than normal rat adrenal Determinations by the Sayers and Saffran techniques on the same material are not yet available The values obtained by Steelman and co workers (1956) on adrenotrophic tumours by the Sayers technique were much lower than those obtained by Cohen Bloch and Celozzi (1957) by a modification of the Saffran technique on different samples Ours was a homologous system (Cohen Bloch and Celozzi 1957) and there is good evidence that pituitary hormones of the same type have some species specificity

Dorfman Another important point is that the malignant tumour responded to ACTH stimulation

Furth The responsive adrenal tumour is highly malignant usually metastasizing to the lungs and frequently to other organs

Gardner Do you think that per unit of tumour tissue the rate of hormone production is greater in the tumours than in the normal pitui tary or do you think from the assays you have done that the storage capacity for hormone in the tumour is greater than in normal tissue? It would be interesting if the rate of production of ACTH in the tumour cell were as great or greater than it would be in a normal pituitary gland The possibility of production and release mechanisms from the cell must also be considered

Furth Production of ACTH will have to be studied by administration of labelled metabolites We have only *in vitro* (storage) values The hormone content of adrenotrophic tumours is much less than that of pituitaries (Steelman *et al* 1956) However all of these radiation induced tumours are autonomous The hormone content of dependent thyrotrophic and mammotrophic tumours approximates to that of the

so nicely It produces obesity tremendous water intake and outflow of urine When however we transplanted this tumour into mice of a subline of the line from which it arose the tumour started to grow very rapidly and the animals failed to show these particular effects If we carry it in what we call an E subline then it shows the same effects that Dr Furth has described I wonder whether there has been in this particular line of host an environment that selected out a different type of tumour cell in a heterogeneous population of cells in the tumour that did not necessarily show the adrenotrophic effects or maybe this one subline of this inbred strain of animals that we have responds differently as far as end organ reaction is concerned We have transplanted this rapidly growing deviant back into the subline and it behaves as if it had been inalterably changed

Dorfman Was that a spontaneous tumour?

Gardner This happened to appear in an animal that had been irradiated with 175 rontgen units However we cannot be sure of its cause

Furth So far all adrenotrophic tumours isolated occurred in irradiated animals The first three strains were induced in LAF_1 mice by an atomic bomb explosion In current studies of the pathogenesis and some genetic aspects of pituitary induction (with Dr R Buffett) we noted that both cyclotron neutrons and X rays would induce them in LAF_1 mice In the field of genetics Prof Gardner s work indicates that there is an unexplored realm with respect to the host factor related not only to the occurrence but also to the character of the tumours

Pincus You seem to attribute the effects of the adrenotrophic tumour to cortisone secretion by the mouse adrenal Is it true that the administration of corticosterone to mice will give all the characteristic effects that you observed particularly the obesity the increased thirst the water retention and so on?

Furth I believe that was done by Prof Kendall very recently but his full report is not yet out Corticosterone is the chief corticoid secreted by the adrenal of mice and rats

Gardner We have tried it but maybe the dose was not right because our animals all died of infections before they attained this particular stage

Furth This is very interesting it shows that corticosterone also enhances infection

Gardner We used cortisone not corticosterone and as I recall it the dose was about 60 µg every other day It was lethal within a few weeks

Crooke Obviously some of the effects of these tumours producing trophic hormones are direct on the target organs but it is possible that some of the effects are indirect We have been interested in such indirect effects in the human subject We have assayed urinary gonadotrophins by two different methods simultaneously namely FSH by the Steelman Pohley type of assay and total gonadotrophins by the mouse uterus response and we have observed a very significant increase in the excretion of FSH and decrease of total gonadotrophins and hence mainly of interstitial cell stimulating hormone in the human under the influence

of cortisone So one feels that the response to a tumour producing ACTH may be partly indirect and due to side effects of the increased corticoid secretion on other pituitary hormones

Furth That adrenotrophic tumours act by way of the adrenals is indicated by adrenalectomy promptly abolishing all secondary changes caused by them The sequence of changes to thyrotrope another monomorphous tumour culminates in a polyglandular insufficiency The thyrotropes appear to have a slight built in gonadotrophic (HFS?) potency The enlarged ovary secretes excessive amounts of oestrogen the latter in turn stimulates secretion of mammotrophin and this in turn the mammary gland Now how do we know that this is the sequence? A graft of TSH secreting tumour in a hypophysecto mized animal causes ovarian and uterine stimulation but not mammary gland hyperplasia Removal of the ovaries will abolish the mammary gland stimulation This is an example of how a single trauma radio thyroidectomy sets in motion a fascinating cycle resulting in a multi hormonal disturbance reminding one of Claude Bernard s principle of determinism

Dorfman The stimulation of your tumours by ACTH brings up some interesting points First there appears to be a discrepancy between the ascorbic acid titres of the ACTH and the ability of this material to stimu late hormone formation by the quartered rat adrenal

Furth The rat adrenal corticoid tumour responds to ACTH better than normal rat adrenal Determinations by the Sayers and Saffran techniques on the same material are not yet available The values obtained by Steelman and co workers (1956) on adrenotrophic tumours by the Sayers technique were much lower than those obtained by Cohen Bloch and Celozzi (1957) by a modification of the Saffran technique on different samples Ours was a homologous system (Cohen Bloch and Celozzi 1957) and there is good evidence that pituitary hormones of the same type have some species specificity

Dorfman Another important point is that the malignant tumour responded to ACTH stimulation

Furth The responsive adrenal tumour is highly malignant usually metastasizing to the lungs and frequently to other organs

Gardner Do you think that per unit of tumour tissue the rate of hormone production is greater in the tumours than in the normal pitui tary or do you think from the assays you have done that the storage capacity for hormone in the tumour is greater than in normal tissue? It would be interesting if the rate of production of ACTH in the tumour cell were as great or greater than it would be in a normal pituitary gland The possibility of production and release mechanisms from the cell must also be considered

Furth Production of ACTH will have to be studied by administration of labelled metabolites We have only *in vitro* (storage) values The hormone content of adrenotrophic tumours is much less than that of pituitaries (Steelman *et al* 1956) However all of these radiation induced tumours are autonomous The hormone content of dependent thyrotrophic and mammotrophic tumours approximates to that of the

normal pituitary With acquisition of autonomy the hormone content (storage) of these tumours drops Production rates and degrees of specific responsiveness and the quality of hormones remain to be studied

Pincus Did you suggest that adrenalectomy produces a much larger content of ACTH?

Furth Yes On theoretical grounds The tumours in adrenalectomized hosts grow at a somewhat greater rate but we did not comparatively study the rates of hormone production There is also a qualitative difference between the tumours grown in intact and in adrenalectomized hosts In intact hosts the tumours are riddled with areas of necrosis in adrenalectomized hosts they attain a much larger size without undergoing degeneration This led us to suppose (as a working hypothesis) that the corticoid may interact with adrenotropes

Huseby Did you get the mammary gland changes of lactation in the absence of oestrogen and progesterone?

Furth There is about as much lactation in ovariectomized females with grafted mammotropes as in intact females with similar tumours of the same size There are milk cysts in the tremendously stimulated breasts if you are unaware of this while doing the autopsy your face may be *full of milk* Some virgin females with mammotropes actually nursed young and one carried them to weaning age The ovaries of tumour bearing rats have lower oestrogen secretion rates than normal rats as judged from the size of their uteri However the adrenals of these tumour bearing animals were not removed but judging from the size of the uteri (and appearance of the ovaries) their total gonadal hormonal secretion was below normal With a better knowledge of co factors the virgin mice could be made splendid foster mothers

Dorfman Are you suggesting Dr Furth that this active material can stimulate the mammary gland without oestrogens progesterone or any other factors?

Furth Yes the hormones of mammotropes do this Some corticoids are needed for their action but not oestrogen

Dorfman In other words corticoids are necessary but gonadal hormones are not?

Furth It seems so in animals receiving the hormones of the mammotropes In normal hosts oestrogens are needed to activate mammotropes and corticoids to enable (permit) them to act

Pincus Your photograph of the mammotrophic tumour showed rather an enlarged adrenal?

Furth The enlargement is due partly to the somatotrophic effect and partly to fatty degeneration It is a mistake to judge adrenotrophic effect by adrenal weights For example amphenone causes a marked enlargement of the adrenals with hypofunction and we found the same in thyrotrophic tumour bearing animals

Leathem These pituitary transplants then have been successful in both sexes so would you get mammary gland growth if the pituitary tumours were transplanted to males?

Furth Yes in male rats but not in male mice The oestrogen levels are we believe higher in male rats and androgens are antagonistic

Leathem I ask this because there is such a trivial mammary tree that if the tumours develop the gland you have grown the complete system

Furth Not entirely We were uninformed enough to try nursing by males The nipple of the male remains undeveloped while the rest of the mammary gland becomes tremendously hyperplastic when stimulated by mammotropes

Gardner A number of years ago Dr White and I found that with pure lactogenic hormone we could not get mammary growth unless we gave oestrogen so I assume that there would still have to be steroid from some source We are talking about mammary growth of the early duct system and not the complete type of mammary growth that you described That is an important point about the difference between the rat and the mouse because the male rat may have as elaborate a mammary gland as the female rat except for the absence of nipples

Furth We should also consider the possibility that prolactin is a degraded hormone and somewhat species specific The idea that early development of the duct system is due to direct action by steroids should also be examined Some corticoids are essential to permit the action of mammotropes How this permissive action operates and which steroids do the operation remain to be learned

Woolley You mentioned that there was mammary gland stimulation after hypophysectomy and gonadectomy but what about the combination of gonadectomy and adrenalectomy? Would there still be pituitary activity of a mammotrophic type?

Furth Mammotrophic activity is much reduced by adrenalectomy but it is still present in hypophysectomized gonadectomized adrenalectomized rats although at a much reduced level

INDUCTION OF PITUITARY TUMOURS AND MELANOMAS IN THE GOLDEN HAMSTER

E S Horning

The Chester Beatty Research Institute London

Introduction

The object of this communication is briefly to describe the induction of cutaneous melanomas in the Syrian golden hamster by treatment with 9 10 dimethyl 1 2 benzanthracene (DMBA) together with the influence of oestrogens on melanogenesis and the possible role played by pituitary lesions in this process

The golden hamsters differ considerably from other rodents in several respects and especially in their response to prolonged oestrogenic stimulation When the male hamster is treated with the female sex hormone 90 per cent and often more develop bilateral and multifocal kidney tumours (Kirkman and Bacon 1949 Horning and Whittick, 1954) This however is a sex limited phenomenon as the intact female does not develop kidney lesions after similar treatment Moreover these renal tumours are hormone dependent as they will only grow as transplants in host animals that have been pretreated with oestrogen (Horning 1954 1956) Occasionally, multiple primary tumours develop in other organs after treatment Another peculiarity is that oestrogen induces tumours of the pars intermedia of the pituitary, whereas in other species of rodents oestrogen treatment invariably induces lesions of the anterior hypophysis (Vasquez Lopez 1944 Koneff Simpson and Evans 1946 Horning 1956) Mature hamsters possess highly pigmented dorsolateral scent glands the melanin deposition of which is under the influence of the sex hormones (Fig 1) Following treatment with stilboestrol (3 4 di p

hydroxyphenylhex 3 ene) the pigment in these glands rapidly diminishes and in some instances is macroscopically absent If a hamster, however is treated with combined doses of testosterone propionate and oestrogen the pigment is maintained Kupperman (1946) has shown that it disappears in immature gonadectomized males These points were em phasized in order to demonstrate the peculiar sensitivity of the golden hamster to oestrogenic stimulation

Experimental

Male golden hamsters 6–8 weeks of age of indeterminate ancestry, bred in the Chester Beatty Institute were used exclusively in these experiments They were divided into four separate groups The first group were treated weekly with a 1 per cent solution of 9 10 dimethyl 1 2 benzanthracene alone The carcinogen was applied with a camel hair brush and the painting was restricted to the surface area of the highly pigmented scent glands The second group were treated in a similar manner with DMBA but in addition they received subcutaneously a 20 mg pellet of pure stilboestrol in their left flanks The third group received a single application of DMBA whilst the fourth and last group were treated weekly with a 0 3 per cent solution of 20 methylcholan threne in benzene (See Table I)

This paper will be concerned only with the first two groups those repeatedly treated with DMBA alone and those with the carcinogen plus stilboestrol

Following the weekly application of DMBA alone to the surface of the pigmented scent gland numerous isolated patches of black pigment begin to appear in the periphery of the gland and they gradually radiate out until they occupy a large area of the dorsal region (Fig 2) It will be noticed that this pronounced scattering of pigment although extensive remains localized in the skin area surrounding the scent gland This peculiar development of pigment invariably occurs from two to four months following the repeated applica tion of the carcinogen As melanogenesis proceeds these

Table I

Number of hamsters treated	Sex	Treatment	Average duration treatment (weeks)	Papillomas or epitheliomas	Number of blue melanomas	Number of survivors over 1 year
20	♂	9 10 Dimethyl 1 2 benzanthracene (weekly)	40	16	14	16
20	♂	DMBA Stilboestrol (weekly)	38	8	0	8
24	♂	DMBA (single application)	46	0	0	8
10	♂	20 Methyl cholanthrene (weekly)	41	0	0	7

numerous individual patches of black pigment increase in size and invariably become raised from the surface of the skin

If we now in comparison examine the dorsolateral region of a hamster from the second group that has been treated weekly with DMBA together with stilboestrol for a similar length of time it will be noticed that the black pigment in the skin area of the scent gland is far less pronounced only several very small patches of pigment being seen (Fig 3)

Let us now revert to group one and examine a hamster that has been treated repeatedly with DMBA alone for over 14 months An epithelioma has arisen at the site of application of the carcinogen obliterating the scent gland and in the periphery of the skin tumour numerous melanotic lesions have developed The remaining untreated scent gland on the other hand is macroscopically normal and no pigment is seen in the surrounding skin area However in an area of the skin remote from the site of application of DMBA a large blue



Fig 1 Dorsolateral pigmented scent glands in a mature male golden hamster



Fig 2 Both the pigmented scent glands have been painted for several months with DMBA Note the development of pigmented patches in skin area of the gland



Fig 3 Scent glands after similar period of treatment (as that seen in Fig –) with both DMBA and stilboestrol Pigment in skin area of glands is much less conspicuous



Fig 4 Epithelioma at site following application of DMBA Note black pigmented lesions in periphery of tumour A large blue melanoma has arisen at a site remote from treatment with the carcinogen

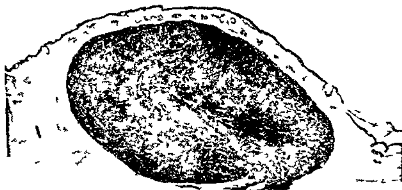


Fig 5 Section through blue melanoma seen in Fig 4



Fig 6 Section through a black melanotic lesion Compare with Fig 5



Fig 7 Macroscopic photograph of a pituitary tumour of the pars intermedia in a male hamster following prolonged treatment with stilboestrol



Fig 8 Section through a tumour of the pars intermedia after progressive invasion of both the anterior and posterior lobes The intermediate cells can be seen undergoing degenerative change

melanoma has arisen (Fig 4) [Out of a total of 16 hamsters which survived weekly treatment with DMBA alone for over one year 14 developed blue melanomas at a site remote from treatment Either epitheliomas or papillomas together with black melanotic lesions at or near the site of tumour formation, also arose in every instance Although the melanotic lesions which arise in the skin remote from the site of application of the carcinogen are termed 'blue melanomas , as distinct from the smaller black pigmented lesions which develop in the skin adjacent to the treated scent glands, this does not imply that the blue lesions contain a different kind of pigment from those observed in the black lesions The blue colour is imparted to the skin by the fact that the melanin is seen through a greater thickness of tissue (Fig 5) Although these blue lesions appear to be encapsulated in some instances the melanotic cells can be seen invading the muscle of the host whilst in the so called smaller black lesions the melanin in the epidermis is very diffuse (Fig 6)

These so called blue melanotic lesions are similar to those recently obtained by Porta and co workers (1956) with DMBA in the hamster They however treated large areas of the dorsal skin whilst in these experiments painting with the carcinogen was restricted solely to the pigmented scent gland

It is proposed to confine this paper to the endocrine control of pigmentation and not to discuss the histogenesis of these lesions nevertheless it may be appropriate at this juncture to suggest that the black lesions appear to arise in the epi dermis whilst the more deeply seated blue melanomas appear to be dermal in origin Studies of the histogenesis of these lesions are still in progress and will later be made the subject of a separate report For the sake of convenience we shall now study the results of the experiments illustrated in Table I, in which we shall see the differences in response between the incidence of blue melanomas induced in the hamsters treated with DMBA alone and those simultaneously treated with the carcinogenic hydrocarbon and stilboestrol Fourteen

melanomas developed in those treated with DMBA alone and none arose in the 20 which received the combined treatment

Owing to the possible implication of the pituitary in this phenomenon, it might be advantageous before passing on to the interpretation of these results to discuss briefly the types of pituitary lesions which arise in the hamster following oestrogenic stimulation

Vasquez Lopez in 1944 first described the induction of tumours of the pars intermedia of the pituitary in the golden hamster following treatment with both natural and synthetic oestrogens In 1946 his results were confirmed by H M Evans and his associates (Koneff Simpson and Evans)

The intermedia undergoes hyperplasia and invades progressively the pars anterior and posterior as well as the infundibulum (Fig 7) In contrast to other rodents after similar treatment no marked histological changes occur in the anterior lobe other than a rapid infiltration of it by the pars intermedia In some animals after 6–8 months treatment both the posterior and anterior lobes have become completely infiltrated by the intermedia cells After this has occurred the invading cells undergo marked degenerative changes, involving vacuolation, fragmentation and in most instances disintegration of the nuclei (Fig 8)

Discussion

It is well known that certain endocrines play an important role in regulating the skin pigmentation of vertebrates We are familiar with the pronounced pigmentation seen in adrenocortical hypofunction (Addison s disease) and the increase in skin pigment that occurs during pregnancy Likewise the clinical administration of oestrogens or corticotrophin invariably induces a similar condition but cortisone does not

The *evidence that the pituitary elaborates and secretes* a melanocyte stimulating hormone (MSH) whose target organs are the pigment cells of the skin has been obtained mainly from animal experimentation Early experiments revealed that hypophysectomy in fishes amphibians and reptiles leads

to a diminution of skin pigment, whilst injection of pituitary extract causes a rapid darkening of the skin It is now firmly established that increased skin pigmentation is due to a dispersion of the melanin within the melanophore under the influence of intermedin The recent experiments of Dalton and Krassner (1956) are of interest Working on the pituitary influence of pigment pattern development in the white axolotl they showed that the sources of new melanophores are dependent upon pituitary activity They also demonstrated that the immediate effect of hypophysectomy is the curtailment of pigment cell production by differentiation of melanoblasts

MSH in most animals is elaborated by the pars intermedia or in the anterior lobes of those animals which lack a discrete intermediate lobe According to Landgrebe Ketterer and Waring (1955) there is evidence that the intermediate lobe cells in man and in the higher mammals migrate into the anterior lobe and this might be the reason why intermedin is sometimes associated with anterior and posterior lobe extracts According to these workers the problem is complicated by the fact that the pars intermedia probably secretes more than one active substance since a number of properties have been ascribed to extracts rich in melanophore expanding activity

Recently Lerner Shizume and Bunding (1954) have reported darkening in the human skin and the formation of new naevi following administration of a preparation of MSH containing very little corticotrophin or other pituitary hormones Excretion of MSH was increased in patients with Addison s disease and in others after bilateral adrenalectomy They further found that cortisone inhibited excretion of MSH It was also of interest to note that they found decreased amounts of the melanophore hormone in the blood and urine of some patients suffering from decreased pituitary function

It might well be that continuous oestrogen administration by causing decreased pituitary function in the hamster depresses the melanophore hormone in a similar way to that Lerner Shizume and Bunding (1954) have described in patients with decreased pituitary function

The influence of sex hormones on melanophores has been extensively studied by Hamilton (1941) in the chick Explants of the skin ectoderm were grown *in vitro* He reported that *oestradiol and cortexone inhibit the differentiation of* black melanophores, whilst testosterone induces an increase in this pigment In view of the marked reduction of the incidence of melanomas in oestrogenized hamsters with pituitary lesions the inhibitory effect of oestradiol is of interest Hamilton concludes that the hormone acts directly on the melanophores and inhibits the rate of melanin synthesis within the cell

In earlier experiments this worker (1940) found that the action of the cortical hormone is apparently that of a suppressor of melanin formation in the skin of the chick The cortical hormone when applied in tissue cultures not only suppressed melanin formation but also caused degeneration of melanophores and inhibited the differentiation of new ones He also stressed that these results may be of interest in view of the fact that it is clinically observed that Addison s disease is accompanied by a darkening of the skin as though the normal inhibition of pigment formation had been removed with the destruction of the adrenal cortex

In conclusion it appears from these hamster experiments that oestrogen suppresses melanogenesis and that tumours of the pars intermedia of the pituitary may be involved in this process It has been shown first by Vasquez Lopez (1944) then by Koneff Simpson and Evans (1946) as well as by the *present author (1956) that prolonged oestrogenic stimulation* induces first a hyperplasia and then an infiltration of the pars intermedia into both the anterior and posterior lobes of the organ Furthermore this progressive invasion is then followed by marked degenerative changes in the intermediate cells involving vacuolation fragmentation and in some *instances disintegration of the nuclei Evans further reported* a final destruction of the intermediate cells and their conversion in some cases into herring bodies

It is possible that these destructive changes which occur in cells of the pars intermedia induced by oestrogenic stimula

tion inhibit the elaboration and secretion of the MSH sufficiently to prevent the formation of cutaneous melanomas which are readily induced in hamsters treated with the carcinogenic hydrocarbon alone

As Lerner Shizume and Bunding (1954) have shown that cortisone inhibits the secretion of MSH experiments have been devised to ascertain the influence of this hormone on the production of melanomas in the hamster In order to help elucidate the role played by the hypophysis in melanogenesis in the hamster experiments are in progress in which radioactive pellets are implanted into the pituitary in order to determine if it is possible to induce melanomas with DMBA in the absence of a functioning pituitary

REFERENCES

DALTON H G and KRASSNER Z P (1956) *J exp Zool* 133 241
HAMILTON H L (1940) *Anat Rec* 78 525
HAMILTON H L (1941) *J exp Zool* 88 275
HORNING E S (1954) *Brit J Cancer* 8 627
HORNING E S (1956) *Z Krebsforsch* 61 1
HORNING E S and WHITTICK J W (1954) *Brit J Cancer* 8 451
KIRKMAN H and BACON R L (1949) *Anat Rec* 103 475
KONEFF A A SIMPSON M E and EVANS H M (1946) *Anat Rec* 94 169
KUPPERMAN H S (1946) *Anat Rec* 96 530
LANDGREBE F W KETTERER B and WARING H (1955) Hormones and the Posterior Pituitary *In* The Hormones ed G Pincus and K V Thimann New York Academic Press Inc
LERNER D B SHIZUME K and BUNDING I (1954) *J clin Endocrin Metab* 14 1463
PORTA G DELLA RAPPAPORT H SAFFIOTTI U and SHUBIK P (1956) *Arch Path* (*Lab Med*) 61 305
VASQUEZ LOPEZ E (1944) *J Path Bact* 46 1

DISCUSSION

Leathem Is there any indication of adrenal degeneration in the animals which have been subjected to the long term treatment? I recall looking at hamster adrenals and they seemed to be wired in reverse by lacking lipid which they seemingly acquire under excessive stimulation The secretability of these particular tissues may be different and I am wondering how you might relate them to your findings in the skin We have considerable information on the relation of the adrenal to

pigmentation in other rodents but the hamster with some of its peculiarities seems to present a unique problem of its own

Horning The adrenal cortex undergoes degeneration in some but not in all hamsters and about 5 per cent of those treated with oestrogen though not with DMBA alone develop hyperplasia of the adrenal cortex Experiments are already in progress in which hamsters are being adrenalectomized in order to determine the role played by this organ in carcinogenesis I found that if other rodents were treated with any of these carcinogenic hydrocarbons plus stilboestrol there was a fairly high mortality It is slightly higher for hamsters

Woolley When you speak of degeneration of the adrenal is there any particular zone that is involved?

Horning Degeneration is fairly common in the zona reticularis but I have not yet made an extensive study of this aspect of the problem

Furth I am confused about true melanocyte stimulating pituitary hormones There is no doubt that both ACTH and the intermediate lobe have melanocyte stimulating activity Are there two hormones? It is stated that when the anterior lobe hormone is heated its melanocyte stimulating activity is enhanced Is this true for the middle lobe hormone?

Horning I wish I knew Dr Furth—in the hamster there is no experimental evidence of the existence of two hormones such as you advocate

Woolley On histological evidence there is some overlap of cells in the hamster intermediate and anterior lobes Some cells from the intermediate layer of the hamster stain as if they were anterior lobe cells so some of the cells may exist in both the anterior and in the intermediate lobes

Horning That is a possibility

Crooke The relation of pigmentation to adrenocortical disease is obscure One sometimes sees profound pigmentation occurring in patients with florid Cushing s syndrome it is admittedly rare but it is very difficult to explain I have seen it begin when the disease was well established and progress steadily

Furth It seems clear now why pigmentation follows long continued administration of ACTH The melanocyte stimulating polypeptide is part of the ACTH molecule Oestrogen is thought to inhibit ACTH activity *and some findings described can be explained by such inhibition* On the other hand stilboestrol is very toxic and I wonder whether your second group has fewer tumours merely because of stilboestrol toxicity Malnutrition also inhibits tumorigenesis

Horning The hamsters do not lose weight following prolonged treatment with stilboestrol alone as quickly as rats do We have frequently treated them for two years or more in order to induce kidney tumours *Their tolerance for stilboestrol is remarkable*

Boyland The dose of stilboestrol is small and is released slowly so that it should not be enough to inhibit appetite

Furth We found that large doses of stilboestrol were toxic to rats and mice

Leathem The hamster is much less sensitive We have given 5 mg

of cortisone to hamsters without particularly influencing their growth and peculiarly enough there is very little suppression of adrenal size whereas in rats this would have a very pronounced physiological effect

Dorfman Are there any signs of hypersecretion of ACTH when the MSH is increased in your animals?

Horning I would very much like to go into that problem It has not been done yet as far as I know

Furth Lymphopaenia and eosinopaenia are simple and sensitive indices of hypercorticalism

Boyland Stilboestrol may be only indirectly necessary for the growth of the kidney tumours but it may affect the pituitary causing the secretion of some other hormone which is necessary The tumours may be dependent on a pituitary hormone rather than on stilboestrol It might be worth while to see if these tumours would grow in animals which are treated with pituitary hormones as well as in hamsters treated with stilboestrol

Furth A crucial experiment would be to try to induce kidney tumours in hypophysectomized animals I have been thinking about the pituitary mediation and the question is which pituitary hormone is related to renal growth and function?

Leathem This mixed up pituitary gland could have some secretion and we had Selye s lyophilized anterior pituitary with adrenotrophic action at one time

Horning Experiments are in progress in which we are trying to see if it is possible to induce renal tumours in the absence of a functioning pituitary by implanting yttrium beads in the hypophysis The mortality in preliminary experiments unfortunately is rather high

Furth There is a short cut to studies of the genesis of those tumours which require years for completion Studies of responsiveness of transplanted tumours to various factors may hint at what might influence their genesis In your case the procedure would be to transplant the tumours to normal and hypophysectomized animals and give the animals various hormone supplements

I should like to say a word of caution about the yttrium 90 experiments Ionizing irradiation of the pituitary will induce pituitary tumours but this result is dose dependent About 500 r will act well much smaller doses will not Doses of 1 000 r and more cause atrophy of pituitary and the target organs You may establish the optimum dose better by direct pituitary irradiation than with yttrium 90

Horning This is very interesting information to have

Huseby Once you have isolated dependent transplants of these renal tumours are they dependent solely upon stilboestrol or will natural oestrogens also stimulate their growth?

Horning I have tried oestradiol and oestrone with success but with stilboestrol 100 per cent will sometimes grow whereas with oestrone only about 70 per cent grow

Huseby If there is some stimulation with natural oestrogen then you have another investigative technique at your disposal You can place the explant in the spleen and an oestrogen pellet next to it In this way

pigmentation in other rodents but the hamster with some of its peculiarities seems to present a unique problem of its own

Horning The adrenal cortex undergoes degeneration in some, but not in all hamsters and about 5 per cent of those treated with oestrogen though not with DMBA alone develop hyperplasia of the adrenal cortex Experiments are already in progress in which hamsters are being adrenalectomized in order to determine the rôle played by this organ in carcinogenesis I found that if other rodents were treated with any of these carcinogenic hydrocarbons plus stilboestrol there was a fairly high mortality It is slightly higher for hamsters

Woolley When you speak of degeneration of the adrenal is there any particular zone that is involved?

Horning Degeneration is fairly common in the zona reticularis but I have not yet made an extensive study of this aspect of the problem

Furth I am confused about true melanocyte stimulating pituitary hormones There is no doubt that both ACTH and the intermediate lobe have melanocyte stimulating activity Are there two hormones? It is stated that when the anterior lobe hormone is heated its melanocyte stimulating activity is enhanced Is this true for the middle lobe hormone?

Horning I wish I knew Dr Furth—in the hamster there is no experimental evidence of the existence of two hormones such as you advocate

Woolley On histological evidence there is some overlap of cells in the hamster intermediate and anterior lobes Some cells from the intermediate layer of the hamster stain as if they were anterior lobe cells so some of the cells may exist in both the anterior and in the intermediate lobes

Horning That is a possibility

Crooke The relation of pigmentation to adrenocortical disease is obscure One sometimes sees profound pigmentation occurring in patients with florid Cushing s syndrome it is admittedly rare but it is very difficult to explain I have seen it begin when the disease was well established and progress steadily

Furth It seems clear now why pigmentation follows long continued administration of ACTH The melanocyte stimulating polypeptide is part of the ACTH molecule Oestrogen is thought to inhibit ACTH activity and some findings described can be explained by such inhibition On the other hand stilboestrol is very toxic and I wonder whether your second group has fewer tumours merely because of stilboestrol toxicity Malnutrition also inhibits tumorigenesis

Horning The hamsters do not lose weight following prolonged treatment with stilboestrol alone as quickly as rats do We have frequently treated them for two years or more in order to induce kidney tumours Their tolerance for stilboestrol is remarkable

Boyland The dose of stilboestrol is small and is released slowly so that it should not be enough to inhibit appetite

Furth We found that large doses of stilboestrol were toxic to rats and mice

Leathem The hamster is much less sensitive We have given 5 mg

NORMAL AND ABNORMAL IODINATED COMPOUNDS IN THE SERUM OF SUBJECTS WITH CARCINOMA OF THE THYROID*

JAMSHED R TATA†

National Institute for Medical Research London

ALTHOUGH carcinoma of the thyroid in man is a relatively rare disease with many histological types it is now known that in some cases the neoplastic tissue can acquire function resembling that of normal thyroid tissue It was first shown by Keston and co workers (1942) using radioactive iodine that thyroid carcinoma tissue had the ability to concentrate circulating iodide The iodide concentrating ability of functioning carcinomas a large proportion of which are metastatic in nature is less efficient than that of normal thyroid tissue (McArthur and Cope 1946 Pochin Cunningham and Hilton 1954) The accumulated iodine can be incorporated into an organic molecule in the tissue and then secreted as protein bound iodine in the blood (Frantz *et al* 1944 Pochin Cunningham and Hilton 1954) The activity of the neoplastic tissue is thought to be influenced by alterations in pituitary function as illustrated by studies with thyroidectomy and with the administration of thyrotrophic hormone and thiouracil (Leiter *et al* 1946 Trunnell *et al* 1948 Rawson *et al* 1949 Rall *et al* 1951) However more recently Hilton and co workers (1956) have reported the absence of any clear evidence for the effect of thiouracil

For clinical and physiological aspects of the disease the reader could profitably refer to articles by the following

* The author s personal work was carried out in collaboration with Dr J E Rall and Dr R. W Rawson at the Sloan Kettering Institute for Cancer Research New York during the tenure of a Visiting Research Fellowship from 1954–1956

† Beit Memorial Fellow for Medical Research

it might be possible to demonstrate a direct stimulating effect of the oestrogen in contrast to an indirect one say through the pituitary We have done this in the case of certain testicular tumours with I believe some success

Leathem Is that of very great help to you if located in the kidney?

Huseby It would not help if you put both the explant and the pellet in the kidney but if you put both in the spleen then you can demonstrate a local effect because the natural oestrogen enters the portal circulation and is mostly inactivated by the liver prior to entering the systemic circulation

Horning Hamsters as you know are odd creatures in many ways I wanted to find out why it was that hamsters when treated with large doses of natural or synthetic oestrogen developed kidney tumours whilst other species of rodents did not I thought it might be due to the fact that the liver is unable to inactivate the hormone In order to try and prove this I inserted small oestrogen pellets into the spleen two of these animals developed small cortical lesions in the kidneys which suggested that the liver might not be able to cope adequately with the detoxification of the hormone But that experiment is not really a very satisfactory one because if a small fragment of stilboestrol came in contact with the peritoneum it would by pass the liver

Scowen Do you get gross impairment of renal function in the hamster with stilboestrol such as you do in the guinea pig?

Horning I do not think I can answer that question as I have not treated guinea pigs with stilboestrol

Scowen It might help to tell us what is happening if you got a gross disturbance of renal function first Do you think your carcinogen influences the local action of an oestrogen where you apply it?

Horning It is a very interesting point because quite accidentally I found that if I treated hamsters with 20 methylcholanthrene and then with stilboestrol this considerably reduced the incidence of kidney tumours

Scowen The evidence is very good as far as the vaginal epithelium is concerned using the same carcinogen which would prevent the oestrogen action

Gray About this very high mortality what do your animals in fact die of? Do they die of renal insufficiency as Dr Scowen mentioned or what?

Horning That has not been determined in all instances Many apparently die from enteritis

Gray It seems that their mode of death might be very important if it is considered in relation to the gastric changes

Dorfman High concentrations of cortisol might be one factor

Huseby In the genesis and development of the melanomas have you noted any junctional changes within the epithelium?

Horning I have in some of the black melanomas but not in the blue melanomas It is curious how these blue melanomas develop at a site remote from the application of the carcinogens From preliminary studies I have done on the histogenesis of these melanomas I think that the black ones arise from the epidermis and the blue ones from the dermis

frequently and in larger amounts Chromatographic analysis of ^{131}I labelled products in whole serum of three of the above patients is shown in Fig 1

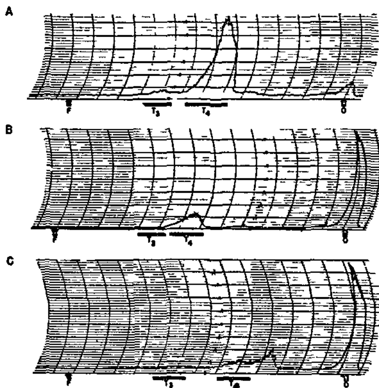


FIG 1 Distribution of ^{1} I labelled substances in serum of three patients (A B and C) with functioning thyroid carcinoma as seen by paper chromatographic analysis Solvent system *n* butanol dioxane ammonia O = origin F = solvent front T = thyroxine T = 3 5 3 tri iodothyronine the ^{131}I peak of R_F just below T is that of iodide The major ^{131}I peaks are A—thyroxine B—thyroglobulin C—Compound X (From Robbins Rall and Rawson 1955)

At the same time in investigations employing zone electro phoresis no difference was observed in the specific thyroxine binding protein (TBP) fraction of serum obtained either from normal or hyperthyroid subjects or from subjects with

authors Seidlin (1952), Rawson Rall and Robbins (1953), Dargent and Berger (1954) Sonenberg and Rall (1956), Sonenberg (1956) Hilton and co workers (1956)

Until the relatively recent work described by Robbins, Rall and Rawson (1953, 1955), little information regarding the chemical nature of circulating iodine compounds elab orated by the functioning carcinoma tissue was available In this report will be presented data gathered from biochemical studies on the nature of serum radioiodine in subjects to whom either tracer or therapeutic doses of ^{131}I were administered

Thyroxine and 3 5 3 tri-iodothyronine in serum of subjects with thyroid carcinoma

In normal and hyperthyroid individuals the serum protein bound iodine is essentially made up of the hormones thyroxine and 3 5 3 tri iodothyronine (Gross and Pitt Rivers, 1951 1952 Rosenberg 1951 Robbins *et al*, 1952 Larson Deiss and Albright 1954) Subjects with carcinoma of the thyroid in the absence of all normal tissue may exhibit a euthyroid status In these subjects organification of iodine continues to take place in metastases and a significant amount of their total blood iodine is protein bound It was hence thought to be likely that both thyroxine and 3 5 3′ tri iodothyronine are present in the blood of patients with thyroid carcinoma In fact chromatographic analysis on the *n* butanol extract of serum from a patient with functioning metastases of the thyroid indicated the presence of both hormones (Gross and Pitt Rivers 1952) At about the same time thyroxine was detected in the serum of athyreotic subjects with functioning metastases distributed in lung neck liver and bone (Robbins *et al* 1952)

Later from a study of 23 patients selected because of highly functional thyroid carcinoma it was established that thyroxine and 3 5 3 tri iodothyronine are present in the circulation (Robbins Rall and Rawson 1955) 3 5 3 Tri iodothyronine was detected in serum in nine out of 15 patients to whom ^{131}I was given and it represented about 1 to 7 per cent of serum organic ^{131}I Thyroxine was detected more

^{131}I in the peak at the albumin zone is due to Compound X * Further resemblance to serum albumin was found in studies

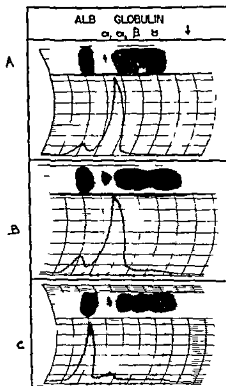


FIG 2 Distribution of ^{131}I labelled substances in serum of three patients (same as in Fig 1) with functioning thyroid carcinoma as seen by zone electrophoresis at pH 8 6 The paper strips stained with bromphenol blue are aligned with records of ^{131}I distribution. The major radioiodine components are A—thyroxine B—thyroglobulin C—Compound X. (From Robbins Rall and Rawson 1955)

* In some cases all the ^{131}I in the albumin region does not uniquely represent Compound X because it has been shown that a small fraction of thyroxine can be bound to human serum albumin The distinction between the two can however be made on the basis of other properties described

thyroid carcinoma (Robbins Rall and Rawson 1955, Horst and Rosler 1953)

It should be noted that as with normal subjects labelled thyroglobulin would be discharged into the blood only when large doses of radioiodine are administered to patients with thyroid carcinoma In fact a large proportion of ^{131}I in the peak seen at the origin of chromatogram B in Fig 1 was found to be serum thyroglobulin by other tests Thyroglobulin in the serum thus released by radiation of the tissue has been found to be similar in nature to thyroglobulin present in normal thyroid tissue (Robbins *et al*, 1952, Robbins 1954 Robbins Petermann and Rall, 1954)

Compound X An abnormal iodinated protein in the serum of subjects with functioning thyroid carcinoma

In their studies on the nature of serum iodine in subjects with functioning thyroid carcinoma, Robbins, Rall and Rawson (1953 1955) reported the presence, besides thyroxine 3 5 3 tri iodothyronine, thyroglobulin and iodide of an unusual organic iodine containing fraction in serum Unlike the two hormones this fraction was only slightly soluble in *n* butanol, immobile in chromatography with acidic and alkaline butanol solvent systems (Fig 1, case C) and was non dialysable The above authors designated the fraction as Compound X This substance has so far only been detected *in the serum of subjects with functioning thyroid carcinoma* The most remarkable property of Compound X is its close association with serum albumin as is seen by its solubility in salt solutions and organic solvents by its complex formation with heavy metal ions and by its electrophoretic mobility Hence it was easily distinguished from thyroxine 3 5 3′ *tri iodothyronine and thyroglobulin in serum by paper* electrophoresis Some illustrative electrophoretic analyses of serum of carcinoma patients treated with ^{131}I are reproduced in Fig 2

In the above patterns serum thyroxine 3 5 3 tri iodothyronine and thyroglobulin move to a position intermediate between that of α_1 and α_2 *globulins while most of the*

protein distinct from albumin For both purposes ¹³¹I labelled HSA was used for comparison with the albumin fraction from the serum of subjects with functioning thyroid carcinoma

Firstly a separation between Compound X ¹³¹I and HSA ¹³¹I was achieved on the basis of the immunologic properties of

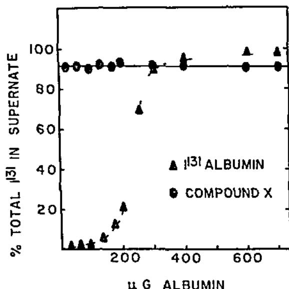


FIG 3 Difference in distribution of ¹³¹I in a precipitin reaction with serum albumin containing labelled Compound X and ¹³¹I tagged HSA Both reactions carried out under identical conditions and with the same anti HSA rabbit antiserum The ¹³¹I curve obtained with iodoalbumin is similar to the one with untagged HSA as measured by other methods
(From Tata Rall and Rawson 1956)

serum albumin A precipitin reaction using serum albumin isolated from carcinomatous patients treated with ¹³¹I, and rabbit anti HSA antiserum easily distinguished the abnormal protein from iodinated or non iodinated serum albumin as illustrated in Fig 3

Just as iodination (up to 10 moles of iodine per mole of

with the ultracentrifuge A value of $S_{20,w} = 4.2-4.8$ for a single iodine containing component was comparable to a value of $S_{20,w} = 4.17$ obtained for serum albumin under similar conditions (Tata, Rall and Rawson 1956 Robbins Rall and Rawson 1955) From these findings it was concluded that Compound X was very likely to be a homogeneous iodinated protein fraction of serum in these subjects

The significance of this abnormal material was realized when it was observed by Robbins Rall and Rawson (1955) to be present in the blood of nearly 60 per cent of the thyroid carcinoma subjects studied That unlike the release of thyroglobulin into serum it was not a product of radiation damage to the neoplastic tissue was demonstrated by the detection of Compound X even when only tracer doses of ^{131}I were administered

Further investigation into the nature of Compound X entailed the isolation of the serum albumin fraction from the blood of patients selected for their rapid rate of fixation of ^{131}I into the abnormal serum fraction The separation was achieved by methods based on properties of solubility and electrophoretic mobility of human serum albumin (HSA), and on the specific property of zinc ions in detaching any bound thyroxine or 3 5 3 tri iodothyronine from the protein (Tata, Rall and Rawson 1956) In all these investigations, the nature of Compound X could only be studied when it was labelled with ^{131}I (because of the minute amounts, 50–100 μg total iodine/litre of blood)

The preliminary failure of commonly employed methods to separate the radioactive moiety from serum albumin suggested that Compound X could possibly be

1 A complex of one or more iodinated compounds of small molecular weight firmly bound to albumin or
2 *An iodinated albumin or*
3 *An iodinated protein distinct from albumin*

Later studies involving two very specific properties of HSA helped in deciding that Compound X was in fact an iodinated

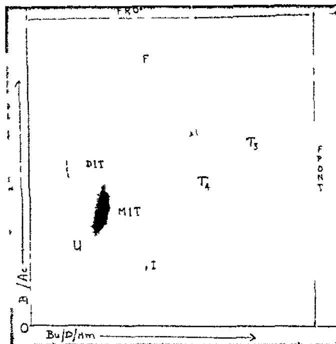


Fig 4 Autoradiography of a two dimensional chromatogram of I labelled Compound X hydrolysate Solvents (1) n butanol acetic acid (Bu/Ac) ( ) n butanol dioxane ammonia (Bu D/Am) The areas surrounded by dotted line represent the positions of substances absent in the hydrolysate (positions known from carrier substances added) I^- = iodide MIT = 3 monoiodotyrosine DIT = 3 5 diiodotyrosine T = thyroxine T_3 = 3 triiodothyronine U and Γ = unknown substances O = origin

(From Tata Rall and Rawson 19)

albumin) failed to alter its immunologic properties (Eisen and Keston, 1949), thyroxine bound experimentally to HSA followed the distribution of serum albumin rather than that of Compound X This method at the same time has proved useful in preparing small quantities of the abnormal iodo protein free from serum albumin (by employing the γ globulin fraction of rabbit anti HSA antiserum)

The second procedure was based on the property of increased thermal stability of serum albumin in the presence of *fatty acid anions (Teresi and Luck, 1952) Thus when Com* pound X in association with serum albumin was heated in the presence or absence of 0 02 M sodium caprylate, a distinction between the two proteins could again be made by measuring the ^{131}I in the precipitate and supernatant fractions (Tata Rall and Rawson 1956)

The protein nature of Compound X was further demonstrated by its susceptibility to hydrolysis by acid alkali and proteolytic enzymes and the subsequent release of both iodinated and non iodinated amino acids (Robbins, Rall and Rawson 1953 1955 Tata, Rall and Rawson, 1956) In Fig 4 is seen one of the results of chromatographic analysis of a pancreatin hydrolysate of isolated Compound X free from the serum albumin fraction

The main iodinated products of hydrolysis are 3 monoiodo tyrosine and an iodinated amino acid having some of the chromatographic properties of thyroxine The absence of 3 5 di iodotyrosine (although small amounts of this compound have been frequently observed) and 3 5 3 tri iodothyronine—iodoamino acids normally present in thyroglobulin—is conspicuous The presence of 3 mono iodotyrosine and the absence or presence only in traces of 3 5 di iodotyrosine as one of the major iodinated components is surprising if one considers that the other major iodinated amino acid is thyroxine For according to the theory of Harington (1944), one molecule of thyroxine in a natural or artificial iodoprotein is derived from the condensation of two molecules of 3 5 di iodotyrosine However, it is possible

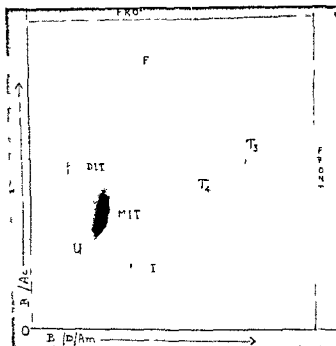


Fig 4 Autoradiography of a two dimensional chromatogram of ^{13}I labelled Compound X hydrolysate Solvents (1) n butanol acetic acid (Bu/Ac) ( ) n butanol dioxane ammonia (Bu/D Am) *The areas surrounded by dotted line represent the* positions of substances absent in the hydrolysate (positions known from carrier substances added) I^- = iodide MIT = 3 monoiodotyrosine DIT = 3 5 di iodotyrosine T = thyroxine T = 3 5 3 tri iodothyronine U and F = unknown substances O = origin
(From Tata Rall and Rawson 19)

that the second major iodinated component of the Compound X molecule is 3 3 di iodothyronine, an amino acid recently shown to be present in thyroglobulin by Roche and co workers (1956) If this is true (although no proof is yet

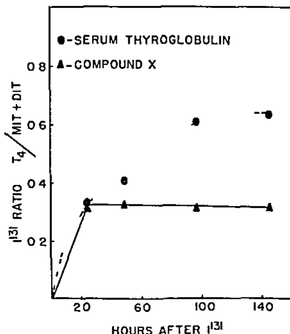


FIG 5 Comparison of rate of incorporation of ^{131}I into the iodothyronine and iodotyrosine fractions of Compound X and serum thyroglobulin isolated simultaneously from the blood of the same subject with functioning thyroid carcinoma T_4 = thyroxine like component MIT = 3 monoiodotyrosine DIT = 3 5 di iodotyrosine

available) then it could be conceived that the condensation of two molecules of 3 monoiodotyrosine to one molecule of 3 3 di iodothyronine occurs in the abnormal protein elaborated by the neoplastic tissue

Data on the rate of incorporation of ^{131}I into iodoamino acids of Compound X and serum thyroglobulin obtained from

adenoid goitre or Hashimoto s disease (Owen and McConahey, 1956 Doniach and Hudson 1957) In both thyroid carcinoma and lymphadenoid goitre a common defect may be responsible for the leakage of an iodinated protein into the blood stream—a defect in proteolysis of the iodoprotein in the tissue possibly combined with a failure of the follicular cells to retain sub stances of a large molecular weight

From the work of Stanley (1956) it appears that an iodo protein resembling Compound X is also present in carcino matous thyroid tissue Here again a high iodine turnover rate is evident when one measures the incorporation of ^{131}I in the abnormal protein isolated from the blood at various time intervals (Tata Rall and Rawson 1956) In addition to this fact the difference in composition between Compound X and serum thyroglobulin present in the same individual (Figs 4 and 5) might be explained on the assumption that the two iodoproteins are elaborated in different cells In any case in thyroid carcinoma a dual defect of protein synthesis and protein storage and retention is visible (One should here consider the fact on the basis of ultracentrifugation studies that Compound X has a molecular weight about one tenth that of 600 000–700 000 estimated for normal thyroglobulin)

On a purely physicochemical basis no explanation can be offered for the absence or presence of only very small amounts of 3 5 di iodotyrosine in the presence of large amounts of 3 monoiodotyrosine in any iodoprotein as is the case with Compound X At this point it would be worth while mention ing other examples where apparently similar natural iodo proteins have been encountered

1 A relative preponderance of 3 monoiodotyrosine has been detected in the iodoprotein present in thyroid tissue of patients with nodular goitre (Pitt Rivers personal com munication 1957) At the same time a more striking similarity was observed between Compound X and the thyroid protein in nodular goitre by comparing the ratio of rates of ^{131}I incorporation in their iodothyronine and

the same subject, have revealed further information of interest A difference was observed in the ratio of ^{131}I in the iodothyronine (thyroxine 3 5 3′ tri iodothyronine) fraction to that in the iodotyrosine fraction (3 monoiodotyrosine 3 5 di iodotyrosine) when the hydrolysates of isolated Compound X and serum thyroglobulin were compared This is shown in Fig 5 where the comparisons were made in function of time following the administration of ^{131}I

With serum thyroglobulin, there was an increase in the relative amount of ^{131}I in thyroxine and tri iodothyronine at the expense of that in the iodotyrosines, in function of time—a situation similar to that observed in normal thyroid tissue On the other hand with Compound X the ratio of iodothyronine ^{131}I/iodotyrosine ^{131}I remained constant over a long period of time No explanation has yet been found for the constancy in this ratio

Discussion

Functional adaptation is a characteristic observed in carcinoma of various endocrine glands With the identification of both thyroxine and 3 5 3 tri iodothyronine in the blood of subjects with thyroid carcinoma incorporation of iodine into the hormone molecule is shown to be a property of both neoplastic and normal thyroid tissue It is rather in the kinetics of iodine metabolism that a more marked difference appears between normal tissue and neoplasia A more rapid iodine turnover and decreased iodine pool are characteristic of thyroid carcinoma (Reynolds Corrigan and Hayden 1953 Pochin Cunningham and Hilton 1954) Although a rapid iodine turnover rate is also observed in thyrotoxicosis subjects with thyroid carcinoma are most often euthyroid and rarely demonstrate any signs of hyperthyroidism

The presence of a circulating iodinated protein is a unique feature of the disease In no other thyroid condition is an iodoprotein found in the blood in the absence of traumatic or radiation damage to the tissue An exception however, is the finding of thyroglobulin in blood of patients with lymph

of non thyroidal tissues especially the mammary gland and was also present in milk (Taurog Potter and Chaikoff 1956 Potter and Chaikoff 1956) Very recently in a case of severe trauma of the human thyroid gland incorporation of ^{131}I into thyroglobulin was found to have stopped at the 3 monoiodotyrosine stage (Costa and Cottino 1957)

3 In biochemical studies on the developing embryonic thyroid gland a 3 monoiodotyrosine rich iodoprotein has been detected at an early stage of the foetal thyroid (Trunnell and Wade 1955) Lastly as one goes down the evolutionary ladder iodinated scleroproteins with 3 monoiodotyrosine as the major iodinated constituent have been found in invertebrates which have acquired the function of concentrating iodine (Roche and Michel 1951)

From the above considerations a basic similarity emerges when the composition of Compound X is compared with some natural iodoproteins In all these cases the substitution of a second atom of iodine in the tyrosyl residue of the iodoprotein is greatly hindered or even totally inhibited It is a matter of speculation whether a common factor is the cause of this defect in synthesis Until this is known one can assume that in the neoplastic thyroid tissue as manifested by the presence of an abnormal iodoprotein there has taken place a biochemical reversion to a form similar to an embryonic stage of function during the development of the normal thyroid tissue

Summary

Neoplastic thyroid tissue can acquire the property of incorporating iodine into molecules of the thyroid hormones thyroxine and 3 5 3 tri iodothyronine Both hormones have been identified in the blood of athyreotic subjects with functioning metastases Moreover in over half of these subjects serum contains an abnormal iodinated protein designated as Compound X this substance is only found in subjects with functioning thyroid carcinoma Some physicochemical properties of Compound X have been described an important

iodotyrosine fractions as illustrated in Fig 6 Unlike normal thyroglobulin the above ratio maintains a constant value over a long period of time It is debatable whether Compound X and nodular goitre thyroglobulin represent the same protein

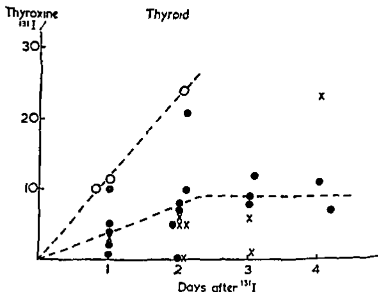


FIG 6 Comparison of rate of incorporation of ^{131}I in the thyroxine fraction of normal human thyroglobulin (O -- O) and thyroglobulin isolated from the thyroid of a patient with nodular goitre (● -- ● × -- ×) Note resemblance to the comparison made between Compound X and serum thyroglobulin in Fig 5
(From Pitt Rivers 1957)

2 Disruption of normal thyroid tissue structure has led to the formation of an iodoprotein with 3 monoiodotyrosine as the major constituent (Taurog Potter and Chaikoff, 1955) Whereas incubation of ^{131}I with thyroid slices gave rise to a labelled thyroglobulin of a normal iodoamino acid composition incubation with thyroid homogenates or their mitochondrial fraction yielded a thyroprotein containing only 3 monoiodotyrosine in the virtual absence of the di iodinated derivative An iodoprotein of almost similar composition was elaborated by slices or particulate fractions

Roche J and Michel R (1951) *Advanc Protein Chem* 4 253
Roche J Michel R Wolf W and Nunez J (1956) *Biochim biophys Acta* 19 308
Rosenberg I N (1951) *J clin Invest* 30 1
Seidlin S M (1952) *Med Clin N Amer* 36 663
Sonenberg M (1956) *Amer J Med* 20 710
Sonenberg M and Rall J E (1956) *Med Clin N Amer* 40 821
Stanley P G (1956) *Biochem J* 63 581
Tata J R Rall, J E and Rawson R W (1956) *J clin Endocrin Metab* 16 1554
Taurog A Potter G C and Chaikoff I L (1955) *J biol Chem* 213 119
Taurog A Potter G C and Chaikoff I L (1956) *Endocrinology* 58 132
Teresi J D and Luck J M (1952) *J biol Chem* 194 823
Trunnell J B Rawson R W Marinelli L D and Hill R (1948) *J clin Endocrin* 8 598
Trunnell J B and Wade P (1955) *J clin Endocrin Metab* 15 107

DISCUSSION

Dorfman In the identification of this compound I presume that the experiments have been carried through with all chemical and radio chemical securities?

Tata Only radiochemical because we are dealing at times with a fraction of a microgram of Compound X I agree that chromatographic analysis is not the final word someone will have to isolate all these products and identify them by classical methods

One indirect method that I did not stress is that we are trying to make Compound X experimentally from normal thyroid tissue Very curiously if the normal structure of the tissue is disrupted a material like Compound X occurs instead of a thyroglobulin of normal composition which our control tissue produced If part of the thyroid tissue is homogenized and either the homogenate or the mitochondrial fraction incubated an iodinated protein is formed which means that the dis rupted tissue has not lost the properties of organification of iodine However if the iodoprotein is hydrolysed only monoiodotyrosine and no di iodotyrosine will be found all experimental conditions being the same These materials can be isolated by radioisotopic dilution and we feel that what we studied in Compound X was something similar

Dorfman You dilute your material out and make derivatives and carry out various chromatographic procedures?

Tata Not always but we are satisfied by constant specific activity over a range of different procedures and different solvent systems

Pincus Is the *in vitro* product also an albumin?

Tata This is difficult to say because when we tried electrophoresing Compound X which was separated from its accompanying albumin it no longer had the same migration which meant that a large amount of albumin was modifying its electrophoretic property as seen in whole

characteristic being high 3 monoiodotyrosine content of this protein in the virtual absence of 3 5 di iodotyrosine The significance of these findings has been discussed

REFERENCES

COSTA A and COTTINO I (1957) *Folia endocrin Pisa* 10 41
DARGENT M and BERGER M (1954) *Bull Ass franç Cancer* 41 344
DONIACH D and HUDSON R V (1957) *Brit med J* 1 672
EISEN H N and KESTON A S (1949) *J Immunol* 63 71
FRANTZ V K BALL R P KESTON A S and PALMER W W (1944) *Ann Surg* 119 668
GROSS J and PITT RIVERS R (1951) *Lancet* 2 766
GROSS J and PITT RIVERS R (1952) *Lancet* 1 439
HARINGTON C R (1944) *J chem Soc* p 193
HILTON G TOCHIN E L CUNNINGHAM R M and HALNAN K E (1956) *Brit J Radiol NS* 29, 297
HORST W and ROSLER H (1953) *Klin Wschr* 31, 13
KESTON A S BALL R P FRANTZ V K and PALMER W W (1942) *Science* 95 362
LARSON F C DEISS W P and ALBRIGHT E C (1954) *J clin Invest* 33 230
LEITER L SEIDLIN S M MARINELLI L D and BAUMANN E J (1946) *J clin Endocrin* 6 247
MCARTHUR J W and COPE O (1946) *J clin Invest* 25 929
OWEN C A and MCCONAHEY W M (1956) *J clin Endocrin Metab* 16 1570
PITT RIVERS R (1957) Personal communication
TOCHIN E L CUNNINGHAM R M and HILTON G (1954) *J clin Endocrin Metab* 14 1300
POTTER G D and CHAIKOFF I L (1956) *Biochim biophys Acta* 21 400
RALL J L MILLER W N FOSTER C G PEACOCK W C and RAWSON R W (1951) *J clin Endocrin* 11 1273
RAWSON R W RALL J L and ROBBINS J (1953) *Arch intern Med* 92 299
RAWSON R W SKANSE B N MARINELLI L D and FLUHARTY R G (1949) *Cancer N Y* 2 279
REYNOLDS L CORRIGAN K E and HAYDEN H S (1953) *J Amer med Ass* 151 368
ROBBINS J (1954) *J biol Chem* 208 377
ROBBINS J PETERMANN M L and RALL J E (1954) *J biol Chem* 208 387
ROBBINS J RALL J E BECKER D V and RAWSON R W (1952) *J clin Endocrin Metab* 12 856
ROBBINS J RALL J E and RAWSON R W (1953) *J clin Endocrin Metab* 13 852
ROBBINS J RALL J E and RAWSON R W (1955) *J clin Endocrin Metab* 15 1315

131I to a patient who has had surgical thyroidectomy you would get the same Compound X as from a patient who has had radiothyroidectomy

Pincus Has there been any attempt to study the enzyme responsible for this condensation?

Tata I do not know if anyone has attempted it I think the best way would be to try and study gorgonines and spongines as models and establish what enzymes are lacking or whether an inhibitor is present and then go on to the human material

Dorfman Did you mention the biological activity of the 3 3 di iodothyronine?

Tata It is slightly lower than that of thyroxine but it has a fairly good activity Very little is known about this compound in mammals It has been studied only very recently and it was synthesized in 1955 One does not know how far this material could be responsible for maintaining the patients in a clinically euthyroid condition

Dorfman Is the reason for the different types of responses for the thyroid known?

Tata No but it is the current fashion now in studying these different compounds to see if they have different actions

Furth You mentioned precipitin reaction as a means of separating albumins from Compound X The gel diffusion techniques of Ouchterlony and of Grabar may be the most sensitive methods for detection of the various kinds of thyroidal albumins in tumours as well as in normal thyroids with or without incubation with TSH Did you employ these techniques?

Tata No but at present we have reached an impasse with Compound X The only method is immunological and we are trying to show that the abnormal material and the normal material have the same immuno reactive grouping Then we can show that what has happened in Compound X is that there is an abnormality in protein synthesis whereby you get Compound X as just a small hunk of thyroglobulin not fully elaborated In other words the molecular weight of thyroglobulin is about 600 000 and that of Compound X is estimated to be about 60 000 on the gel diffusion technique you get two reactive bands with thyroglobulin and it could be that one of them might represent Compound X

Dorfman Has 3 3 di iodothyronine been found to be a product of normal thyroid biosynthesis?

Tata No one has studied this in humans but the studies of Roche and his associates have shown that this compound could be formed in the thyroid gland and also released into the serum of rats They found more of this compound when there was more tri iodothyronine and also at early time intervals in *in vivo* labelling

Dorfman Of course if one only employs chromatography in a single system there may well be instances of contamination of thyroxine with this compound I presume?

Tata Yes It could be that there was some thyroxine present but we cannot explain it since it would be incompatible with Harington s classical theory that thyroxine comes from condensation of two molecules of di iodotyrosine In some of the patients we could not find any di iodotyrosine at all in the iodoprotein hydrolysate

serum What we actually got was a very bad smear with the original *in vitro* substance we got a very large hump spreading all the way out from α_1 and α_2 globulin and albumin but if we added a lot of serum then it all got to albumin

Furth Did you try *in vitro* incubation of tumour slices with and without TSH?

Tata We did *in vitro* incubation but I cannot answer this question conclusively because it was very difficult to get hold of a functioning tumour In humans a lot of tissue was dead tissue and uptake was very low We did not want to go too high with radioiodine because of radiation effects so we used low amounts and it was very rare for us to get any iodoprotein resembling Compound X

Pincus Have you done any studies following hypophysectomy on patients?

Tata No only kinetic studies have been done following hypophysectomy Usually the uptake of radioiodine decreases and there is less serum organic iodine but I do not know if anyone has tried chromatographic studies

Pincus Is there any responsiveness to TSH either in the foetus or in the lower forms which had a di iodoprotein? Isn t it true that thyroid tumours do respond to TSH?

Tata Yes Chaikoff's group have taken hypophysectomized and normal rats and shown that in the normal rat thyroid tissue the iodine uptake decreases when the iodoprotein in hypophysectomized animals is hydrolysed there is a great abundance of monoiodotyrosine and very little di iodotyrosine which can be corrected by giving thyrotrophin

Furth Stable thyroxine should depress hormonal secretion by responsive thyroid tumours Did you study patients after the administration of thyroxine?

Tata No and I must tell you that our studies were based on highly selected patients In other words out of a large number of thyroid cancer patients we got hold of about five who were getting repeated doses of ^{131}I and took them off any other iodinated compound in order that our task would be easy

Furth Not only internists such as Astwood in Boston but also surgeons such as Crile in Cleveland use thyroxine for treatment of functional thyroid tumours This procedure may answer the question as to what TSH does

Tata But there is a limit because you can make these patients hyperthyroid by giving them a lot of thyroxine and many patients with functional carcinoma do not respond well to administration of thyroxine so thyroidectomy is still the solution in such cases

Gardner Have all these patients had large therapeutic doses of ^{131}I?

Tata Yes so there was no normal tissue but only carcinomatous tissue left after a time which was exactly what we wanted

Gardner Do you think that that might have modified the hormone producing capacity?

Tata It increased the tumour s capacity to produce hormones but it was not responsible for the change because if you give tracer doses of

Nevertheless goitrogens alone or combined with a carcinogen have induced thyroid tumours Prolonged feeding of thiourea, thiouracil methylthiouracil and propylthiouracil were effective In rats Purves and Greisbach (1946) observed two thyroid carcinomas in 30 rats given prolonged goitrogen treatment and also noted adenomas in the other animals The tumours were benign adenomas foetal adenomas and adenocarcinomas Animals with adenocarcinomas exhibited multiple lung metastases Money and his co workers (1953) observed a 25 per cent tumour incidence in rats treated with thiouracil for 100 days and this incidence rose to 100 per cent after 500 days Withdrawal of the goitrogen did not enforce a regression of the tumour but the normal thyroid tissue did revert to a normal histology In our experience thiouracil feeding to Long Evans strain rats rarely caused thyroid abnormalities in less than one year but after 15 to 18 months thyroid glands weighing 300 to 500 mg were not uncommon and tumours were observed

Administration of a goitrogen and a carcinogen such as 2 acetylaminofluorene (2AAF) may speed the formation of adenomas increase the frequency of nodules and favour the development of carcinoma (Bielschowsky 1944) Paschkis Cantarow and Stasney (1948) induced thyroid adenomas in 44 of 47 rats The concomitant administration of 2AAF and thiouracil increased tumour incidence 5 of the 7 carcinomas observed were from rats receiving the combination treatment The carcinogen did not influence thyroid weight in the presence of thiouracil despite the increased malignancy but these rats were autopsied at varied times and a re study was suggested Young adult male rats of the Wistar strain were fed 0 1 per cent thiouracil 0 03 per cent 2 acetylaminofluorene or both for periods of 2 4 6 and 8 months Food intake was controlled by paired feeding The carcinogen alone did not influence thyroid weight However the goitrogenic action of thiouracil was significantly enhanced by 2AAF and a weight effect was noted at each time interval (Table I) Relating thyroid weight to body weight revealed that gland sizes were identical in

GOITROGEN-INDUCED THYROID TUMOURS

JAMES H LEATHEM

Bureau of Biological Research Rutgers University New Brunswick

SPONTANEOUS thyroid gland tumours occur only infrequently in rats and mice and thus these species are suitable for a study of experimentally induced tumours Circumstances which favour thyroid tumour formation are associated with prolonged secretion of hypophysial thyrotrophic hormone in excessive amounts The thyrotrophic hormone causes thyroid hypertrophy then hyperplasia which is followed by local cell proliferation adenoma and carcinoma (Bielschowsky, 1955) Thyroid hypertrophy may or may not occur when the thyroid gland is transplanted to the spleen (Rupp 1952) but is evident when a thyrotrophin secreting hypophysial tumour is grafted into a host (Furth, 1955) Thyroid gland hypertrophy can also be invoked with low iodine diets and after a year thyroid tumours may develop (Axelrad and Leblond 1955 Taylor and Poulson, 1956) However, a more marked hypertrophy to 100 times normal weight can be induced with goitrogens and the goitrogens have been combined with other treatment to induce thyroid tumours For example thyroid tumours have been induced by feeding methylthiouracil and injecting a suspension of normal thyroid tissue twice weekly one anaplastic thyroid carcinoma resulted (Teir Carpen and Carpen 1956) In addition an increased incidence of thyroid tumours can be obtained by combining low doses of ^{131}I with a goitrogen Doniach (1953) administered the isotope together with methylthiouracil after 15 months thyroid carcinomas formed in rats given the combination treatment but not in animals receiving only the goitrogen Thyroid gland response to a goitrogen decreases however with an increase in time following ^{131}I as there is an associated reduction in mitoses (Doniach and Logothetopoulos 1955)

1951) The combination of methylthiouracil and 2AAF did not increase the low degree of malignancy of rat thyroid *tumours even if metastases were observed the transplant* grew to a large size without killing the host (Bielschowsky *et al* 1949)

Goitrogens induce thyroid hypertrophy in strain A, C57 and C3H mice (Gorbman 1946 Morris Dubnick and

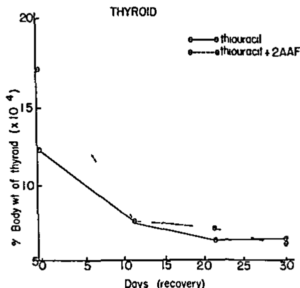


FIG 1 Post treatment regression of thyroid weight in rats fed thiouracil (0 1 %) alone or in combination with 2 acetylaminofluorene (0 03%)

Dalton 1946) The high levels of TSH cause an initial hyper trophy of the follicular epithelium then a follicular hyper plasia which can obliterate the lumen Thyroid weight increases to as much as 25 times normal in the absence of neoplasia have been noted after 12 months of thiouracil feeding Thiouracil ingestion for more than one year was followed by the return of some colloid containing follicles but thyroid tumours could be induced in 16 to 18 months (Morris and Green 1951 Moore Brockney and Bock 1953)

Table I

INFLUENCE OF 2 ACETYLAMINOFLUORENE (0 03%) AND THIOURACIL (0 1%) ON THYROID WEIGHT IN PAIR FED MALE RATS

Days	*Control*	*2AAF*	*Thiouracil*	*2 AAF+ Thiouracil*
60	12 0	13 1	43 2	58 7
120	11 9	13 1	30 7	64 6
180	12 6	13 1	39 5	62 6
240	13 7	12 6	56 1	117 8

Actual thyroid weight in mg (10 rats per group)

control and carcinogen fed rats after 8 months being 4 8 mg per 100 g body weight Thiouracil feeding for 8 months increased relative thyroid weight to 17 8 mg per 100 g, whereas a combination of thiouracil and the carcinogen increased the average gland weight to 38 4 mg per 100 g After 8 months polyp like formation was evident in the thyroid follicles and alkaline phosphatase was increased

Thiouracil withdrawal following goitrogen feeding for 30 to 60 days reverts thyroid weights to normal Thiouracil and a carcinogen together cause an enhancement of thyroid weight and examination of the rate at which the gland weight reverts to normal could possibly characterize a change induced in the thyroid by the carcinogen One unsuccessful test has been performed in trying to detect a thyroid change induced by one month s treatment Young adult male rats were fed 0 1 per cent thiouracil and 0 03 per cent 2AAF or the goitrogen alone After 30 days rats fed the combination of drugs had the larger thyroids Drug withdrawal and examination of the thyroids at various times up to 30 days revealed a similar regression pattern in both groups (Fig 1)

A rat thyroid tumour was found to be transplantable after 23 months of methylthiouracil feeding On transplantation the thyroid tumour was dependent upon a thiouracil environment but when serially transplanted the tumour became a highly malignant neoplasm (Purves Greisbach and Kennedy,

third to ninth transplant and several lines of both dependent and independent tumours have been developed (Morris, 1955)

The C3H male mouse can be used and a varied morphology may be seen at transplantation Failures were obtained in the absence of thiouracil when the transplant morphology resembled hyperplastic thyroid However tissue transition was observed as follicular wall thickening papillary formation and the appearance of abnormal nucleoli Later, colloid formation and follicular structures disappeared a trend towards anaplasia resulted in the appearance of solid sheets of cells New cell types appeared Sarcoma formation was widespread with occasional giant cell and/or cartilage formation The sarcomas were transplantable

Transplantable thyroid tumours may depress the weight of the host thyroid despite goitrogen administration (Lipner Wagner and Morris 1954) The depressing effect upon the host thyroid became apparent as the tumour increased in size suggesting thyroid tumour use of TSH Similar results have been reported with Walker 256 tumour transplants to thiouracil fed rats in which a goitrogenic response to thiouracil was reduced in the presence of the tumour (Begg and White 1956) We have repeated the study using the Walker 256 tumour in rats fed 0 1 per cent thiouracil in a 20 per cent casein diet The animals were fed for two weeks and paired feeding was used to provide a uniform food intake Actual thyroid weight increased from 10 7 mg in control tumour bearing rats to 16 8 mg in thiouracil fed rats and to 15 2 mg in tumour bearing thiouracil fed rats A second study also failed to reveal a tumour depressing effect on the goitrogenic action of thiouracil

It is well known that the thyroid gland can concentrate ^{131}I to amounts well above those in the serum This capacity to concentrate and to organically bind ^{131}I is less than normal in many thyroid tumours (Doniach 1950) In general the thyroid tumours fall into three categories (1) those retaining a capacity to concentrate and bind ^{131}I (2) those lacking the capacity to concentrate and bind ^{131}I and (3) those with

Concomitant administration of 2AAF and thiouracil increased the incidence of thyroid tumours in rats and may shorten tumour induction time A similar study in mice failed to reveal an enhancement of goitrogen action by the carcinogen (Gorbman, 1946) Mice are more resistant to hepatoma induction by 2AAF than rats but some reduction of this resistance can be gained by feeding the drug in a purified diet containing 24 per cent casein and 10 per cent fat (primarily corn oil) A re study of the carcinogen and goitrogen effect on the thyroid was made in Swiss mice Propylthiouracil (0 05 per cent) 2AAF (0 1 per cent) or a combination of these drugs was incorporated in the purified diet and fed for six months to compare with untreated mice Average thyroid weight increased roughly four times in propylthiouracil fed mice However the carcinogen did not enhance the action of the goitrogen, in fact a significantly smaller response was obtained Furthermore the carcinogen alone had a tendency to favour an increase in thyroid weight (Table II) No thyroid neoplasia was observed but alkaline

Table II

INFLUENCE OF 2 ACETYLAMINOFLUORENE (0 1%) AND PROPYLTHIOURACIL (PTU) (0 05%) FOR 6 MONTHS ON THYROID WEIGHT IN SWISS MICE

Treatment	*No Mice*	*Thyroid wt*	
		mg	*mg/10 g body wt*
Control	8	4 8	1 6
2AAF	10	7 0	3 1
PTU	8	18 2	6 4
2AAF+PTU	6	14 2	4 9

phosphatase reduction which is imposed by goitrogen alone was largely counteracted in the presence of 2AAF

Thyroid tumours have been produced by transplanting hyperplastic thyroid tissue from C3H mice treated with thiouracil for three to six months into thiouracil treated hosts Growth autonomy of thyroid was obtained after the

third to ninth transplant and several lines of both dependent and independent tumours have been developed (Morris 1955)

The C3H male mouse can be used and a varied morphology may be seen at transplantation Failures were obtained in the absence of thiouracil when the transplant morphology resembled hyperplastic thyroid However tissue transition was observed as follicular wall thickening papillary formation and the appearance of abnormal nucleoli Later colloid formation and follicular structures disappeared a trend towards anaplasia resulted in the appearance of solid sheets of cells New cell types appeared Sarcoma formation was widespread with occasional giant cell and/or cartilage formation The sarcomas were transplantable

Transplantable thyroid tumours may depress the weight of the host thyroid despite goitrogen administration (Lipner Wagner and Morris 1954) The depressing effect upon the host thyroid became apparent as the tumour increased in size suggesting thyroid tumour use of TSH Similar results have been reported with Walker 256 tumour transplants to thiouracil fed rats in which a goitrogenic response to thiouracil was reduced in the presence of the tumour (Begg and White 1956) We have repeated the study using the Walker 256 tumour in rats fed 0 1 per cent thiouracil in a 20 per cent casein diet The animals were fed for two weeks and paired feeding was used to provide a uniform food intake Actual thyroid weight increased from 10 7 mg in control tumour bearing rats to 16 8 mg in thiouracil fed rats and to 15 2 mg in tumour bearing thiouracil fed rats A second study also failed to reveal a tumour depressing effect on the goitrogenic action of thiouracil

It is well known that the thyroid gland can concentrate ^{131}I to amounts well above those in the serum This capacity to concentrate and to organically bind ^{131}I is less than normal in many thyroid tumours (Doniach 1950) In general the thyroid tumours fall into three categories (1) those retaining a capacity to concentrate and bind ^{131}I (2) those lacking the capacity to concentrate and bind ^{131}I and (3) those with

reduced capacity to concentrate but in which organic binding does occur

Purves, Greisbach and Kennedy (1951) found that a thyroid tumour which was dependent upon a thiouracil environment on transplantation was nevertheless able to concentrate ^{131}I although in subnormal amounts However, organic binding did occur and thyroid hormone was secreted for hypophysial cytology of the thyroidectomized rat was returned to normal The 24 hour ^{131}I uptake by thyroid glands was compared in rats given thiouracil for ten days and for periods sufficiently long so that gland size was abnormal The ^{131}I uptake per mg of tissue was reduced by goitrogen feeding Long term thiouracil administration decreased ^{131}I uptake further in two of three glands (Money *et al*, 1953) Radioautographs of the glands following prolonged thiouracil treatment showed the isotope to be present in atypical follicles and in adenomas but to be lacking in non neoplastic tissue Normally, thiouracil treatment would favour a reduced ^{131}I uptake and an interference with organic binding The tumours therefore may have abnormal iodonating mechanisms or may not be influenced by the thiouracil

In man, significant differences in ^{131}I concentration have been noted in the same nodule despite a similar morphology Furthermore loss of iodonating mechanisms does not parallel the degree of malignancy A highly malignant giant cell or spindle cell carcinoma does not concentrate ^{131}I whereas a much less malignant papillary carcinoma also has very little ^{131}I concentrating capacity (Fitzgerald 1954)

Thyroid tumours concentrate less radioiodine per mg of tissue than does the host thyroid Morris (1955) has shown that a dependent tumour may have half the capacity of the host thyroid to concentrate ^{131}I whereas a very rapidly growing independent tumour may have almost no concentrating capacity (1/1300) More thyroxine was formed by the dependent tumour Paper strip chromatograms have revealed monoiodotyrosine di iodotyrosine and thyroxine in some thyroid tumours as well as in normal glands However

the rate of synthesis in a thyroid tumour may be unusually slow (Berg and Gorbman, 1954) and the presence of a tumour can influence the distribution of monoiodo and di iodotyrosine and thyroglobulin (Scott and Daniel 1956)

A major loss of radioiodide concentration ability accompanies the transition of the tumour from a dependent to an independent status and may be due to a low ^{131}I uptake in the tissue (Wollman 1953) The thyroid/serum (T/S) radioiodide ratio has also been used to study thyroid tumour function The T/S ratio was lower in the tumour than in the host thyroid and was lower in independent than in dependent tumours When organic binding was interfered with by propylthiouracil administration the T/S ratio decreased in the host thyroid and in the dependent tumour but was unchanged in the independent tumour

Administration of thyrotrophic hormone may or may not influence a thyroid tumour However the hypophysial effect on some transplantable tumours in the mouse was indicated by the decrease in T/S ratio of host thyroid and tumour following hypophysectomy (Wollman Scow and Morris 1954) Furthermore thyroid metastases in thyroidectomized patients may respond to ingested thiouracil with an increased ^{131}I uptake and the appearance of thyroxine and tri iodo thyronine in the serum (Rawson and Rall 1955)

In general the evidence at present indicates that the biochemical functional characteristics of a normal thyroid gland are decreased or lost when the gland becomes tumorous

Acknowledgement

Original research incorporated in this report was supported by the American Cancer Society through Institutional grants

REFERENCES

AXELRAD A and LEBLOND C P (1955) *Cancer N Y* 8 339
BEGG R W and WHITE A V (1956) *Cancer Res* 16 554
BERG O and GORBMAN A (1954) *Cancer Res* 14 232
BIELSCHOWSKY F (1944) *Brit J exp Path* 25 90
BIELSCHOWSKY F (1955) *Brit J Cancer* 9 80

BIELSCHOWSKY F GREISBACH W E HALL W H KENNEDY T H and PURVES H D (1949) *Brit J Cancer* 3, 541
DONIACH I (1950) *Brit J Cancer* 4, 223
DONIACH I (1953) *Brit J Cancer* 7, 181
DONIACH I and LOGOTHETOPOULOS J H (1955) *Brit J Cancer* 9 117
FITZGERALD P J (1954) Brookhaven Symposium in Biology No 7 Brookhaven National Laboratory N Y
FURTH J (1955) *Recent Progr Hormone Res* 11 221
GORBMAN A (1946) *Cancer Res* 6 492
LIPNER H J WAGNER B P and MORRIS H P (1954) *Fed Proc* 13 465
MONEY W L FITZGERALD, P J GODWIN J T and RAWSON R W (1953) *Cancer N Y* 6 111
MOORE G E BROCKNEY E L, and BOCK F G (1953) *Proc Soc exp Biol N Y* 82 643
MORRIS H P (1955) *Advanc Cancer Res* 3 51
MORRIS H P DUBNICK C S and DALTON A J (1946) *Cancer Res* 6 492
MORRIS H P and GREEN C D (1951) *Science* 114 44
PASCHKIS K E CANTAROW A and STASNEY J (1948) *Cancer Res* 8, 257
PURVES H D and GREISBACH W E (1946) *Brit J exp Path* 27 294
PURVES H D GREISBACH W E and KENNEDY T H (1951) *Brit J Cancer* 5 301
RAWSON R W and RALL J E (1955) *Recent Progr Hormone Res* 11 284
RUPP J J (1952) *Endocrinology* 51 306
SCOTT K G and DANIEL M B (1956) *Cancer Res* 16 784
TAYLOR S and POULSON E (1956) *J Endocrin* 13 439
TEIR H CARPEN E and CARPEN I (1956) *Acta endocr Copenhagen* 22 395
WOLLMAN S H (1953) *Tex Rep Biol Med* 11 775
WOLLMAN S H SCOW R O and MORRIS H P (1954) *J nat Cancer Inst* 14 593

DISCUSSION

Muhlbock Have you found any difference between male and female animals?

Leathem No we have not It was preferable to use male C3H animals as they could be kept going for a longer time and the mammary incidence was reduced

Boyland Have you repeated Begg s observations with the Walker tumour itself?

Leathem Yes we have but quite unsuccessfully even when we used different diets and regimens In fact our animals which have been fed

thiouracil in the presence or the absence of the Walker tumour as such come out as if there had been no tumour present whatsoever The tumour itself was growing very rapidly Over the 14 day period it had grown to over 20 g and we felt it desirable to terminate the experiment although Begg carried his rats for 20 odd days We also tried the Flexner Jobling tumour equally unsuccessfully We tried both with *ad libitum* and with paired feeding feeling that possibly improved food consumption would make a difference but this did not resolve the problem I do not know quite where we differ from Begg s experiments

Pincus Is the thiouracil dependency direct or indirect?

Leathem The thyroids of animals without the pituitary will not grow of course so we think it is the effect of thiouracil on the pituitary There is then the increased TSH release which is necessary to keep the tissue stimulated and hyperplastic so the result is that it can get completely out of bounds I am sure Dr Furth can see this with his pituitary tumours if he examines thyroids when he transplants at different stages With hypophysectomy of course the tumour growth is remarkably revealing

Furth Hypophysectomy will reduce growth to the level of autonomy TSH dependent thyroid tumours will cease growing Your statement that there is a large area of changes is well borne out by radioautography In stimulated thyroids and multiple adenomas one finds great variability in ^{131}I uptake I wonder whether you thought of using irradiation as a co carcinogen This is an urgent problem at present The induction of thyroid tumours in children was reported by Simpson Hempelmann and Fuller (1955 *Radiology* 64 840) by X radiation of the thymus areas in childhood This was in the Great Lake region in which goitres are prevalent Possibly ionizing irradiation acted as a co carcinogen as in the experiments of Doniach

Leathem We have not done this but it is a very real and necessary point to be studied

Pincus Maybe Dr Money s rats were closer to radiation sources!

Leathem He got an extremely high incidence which is very different from ours Frankly I wish we could produce a 25 per cent incidence of tumours in 100 days because it would be helpful in obtaining the tissues for study

Boyland Regarding the nomenclature I should have thought that if it produced the mutation the irradiation was the initiator and the thiouracil the co carcinogen

Furth This is probably correct Irradiation can produce a silent irreversible change and nothing may happen until a second stimulus is given the latter need not be carcinogenic

Leathem Is there not some information which indicates that during the period immediately after doses of radioactive iodine are administered the response to the goitrogen is actually less than it was initially? The combination of radioiodine and goitrogen at first seems to do a great deal more than if they were spaced out Of course I do not know that this helps us to decide which is the co carcinogen

Gardner The quantitative aspects that you have mentioned are very

interesting I wonder if there are tumours that would grow in the presence of a normal amount of thyroxine such as might be produced by the animal s own thyroid but which would not grow if the hosts received additional thyroxine Has that ever been done? Would it be a desirable thing to do from the standpoint of spreading the extent of those variations of dependency that you mentioned?

Leathem I believe Purves found a dependent tumour in animals which were being fed thiouracil and were therefore dependent but the tumour was elaborating enough thyroxine to keep pituitary cytology normal In fact it controlled itself as it were but we have not done this

Furth Didn t Sellers find that the administration of some thyroxine enhanced tumorigenesis? Furthermore the greatest incidence of pituitary tumours was in those animals which had also received a small quantity of thyroxine

Leathem Yes but there has been some little criticism about the actual content of his diet He was feeding them thyroid powder I think but he may have given thyroxine Someone raised the question of the importance of the amount of iodine which one was offering therefore as a dietary supplement and whether this was really part of the problem But recently Teir Carpen and Carpen (1956 *Acta endocr Copenhagen* **22** 395) have shown that if you administer the goitrogen and inject a suspension of rat thyroid tissue intraperitoneally twice a week you can get thyroid tumours by this means Now does this mean we are giving the thyroid hormone and that thyroxine is really helping the process along? It is an interesting thing to ponder on

Dorfman Will the continuous administration of thyrotrophin produce these tumours?

Leathem I do not really know My experience with TSH as available to us for laboratory use is that it is usually not very active We have unsuccessfully tried 1 mg a day of TSH obtained from a pharmaceutical house

Furth Thyroid tumours are common with autonomous and highly secretory thyrotrophic tumours Occasionally there is metastasis to lymph nodes and invasion of blood vessels

Leathem Your pituitary tumour is doing far better than when we obtain TSH in a vial and inject it

Furth The blood TSH levels in mice with dependent thyrotrophic tumours were estimated by Bates to be about a thousand times normal with autonomous tumours the blood levels are lower but still very high

Leathem The potential protein androgenic possibilities must also be considered

Gardner Do you think that the sodium potassium chloride balance in the diet according to the experiments of Dr Leblond might account for some of the differences in the thyroid responses?

Leathem Nutritionally yes We have not worked on this but certainly inferences from nutritional sources would suggest this I think Leblond s data are fairly good and I wish we had paid more attention to them when we set up long term lines Of course we have purposely tried to use a standard *semipurified type of diet* because when we looked at two

batches of purine foxchow for iodine content we found 15 000 times difference between one batch and another

Furth Two widely used brands of baby food (cereal) were found by Middlesworth to be highly goitrogenic in mice The changes in both thyroid and pituitary were comparable to those of mice massively treated with thiouracil One company hearing about this corrected the deficiency by the addition of iodine

Horning Do these thyroid tumours undergo anaplastic changes? Very often the change from dependency to autonomy is associated with ana plasia which decreases the ability of the cell to produce hormones

Leathem Yes there are anaplastic changes which are quite apparent

BIOSYNTHESIS OF STEROIDS IN HYPERACTIVE AND TUMOUR BEARING HUMAN GLANDS

RALPH I DORFMAN

Worcester Foundation for Experimental Biology Shrewsbury Massachusetts

OUR knowledge of steroid hormone metabolism has been advanced considerably by studies of body fluids from patients bearing hyperactive endocrine glands and functional tumours Many of the known steroid hormone metabolites were discovered by their isolation from urine of patients bearing these hyperactive glands and tumours During the past few years studies of biosynthetic potentials of tissues have been made possible by the development of precision chromatography radiochemical techniques, and methods for the positive identification of microgram quantities of steroids These new methods have now been applied to studies involving hyperactive endocrine glands and functional tumours It is the results of these new studies that will be reviewed against the background of the current hypotheses of biosynthetic pathways

Gonadal hyperactivity and tumours

Steroid biosynthesis of gonadal tissues (Fig 1) seems to follow a pattern involving cholesterol formation from acetate cholesterol transformation to pregnenolone pregnenolone oxidation by means of the enzyme 3β ol dehydrogenase to progesterone 17α hydroxylation of progesterone, and oxidative removal of the side chain of 17α hydroxyprogesterone to yield the C_{19} steroid Δ^4 androstene 3 17 dione and the two carbon residue acetic acid (Slaunwhite and Samuels 1956 Lynn 1956 Lynn and Brown 1956 and Savard *et al* 1956*a* 1956*b*) Testosterone biosynthesis involves one further step the reduction of the $C_{(17)}$ ketone group of Δ^4 androstene 3

17 dione to the 17β hydroxyl group This then is the expected route to the androgens and inherent in the scheme is the bio synthetic pathway to the progestational hormone progester one Oestrogens are biosynthesized from androgens (Baggett *et al* 1956 Heard Jellinck and O Donnell 1955 and West *et al* 1956) so that the over all biosynthetic pathway in cludes all three steroid sex hormones

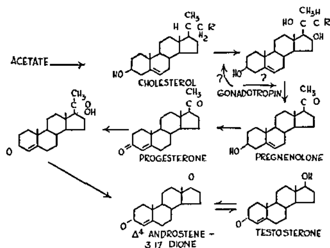


FIG 1 Pathway of androgen biosynthesis in the gonads and to a limited extent in the adrenal cortex

Against this biosynthetic pathway of sex hormones we may discuss some studies involving various types of gonadal tumours Venning Hoffman and Browne (1942) studied the urine of a patient who was diagnosed as having a malignant interstitial cell tumour of the testis and found that the greatly elevated urinary 17 ketosteroid titre (approximately 1 000 mg / day) was due mainly to androsterone and aetiocholanolone and that no proportionately increased quantity of dehydro *epi*androsterone could be found This observation is consistent with the idea that dehydro*epi*androsterone is exclusively an adrenal product Recently we had the opportunity to study the urinary steroids from a 5½ year old boy with an elevated

urinary 17 ketosteroid and whose diagnosis was interstitial cell tumour of the testis An increased concentration of 11 oxygenated 17 ketosteroids was found in the urine When the tumour tissue in the form of slices was incubated with [4 ^{14}C]testosterone in human serum containing added fumarate glucose, equine and chorionic gonadotrophin and pituitary follicle stimulating hormone for 3 hours at 37°C under 95 per cent O_2 5 per cent CO_2 three 11 oxygenated products were demonstrated These included 11β hydroxytestosterone 11β hydroxy Δ^4 androstene 3 17 dione and adrenosterone (Savard *et al*, 1956*a*) After incubation of the same tissue with [4 ^{14}C]progesterone, radioactive 17α hydroxyprogesterone, Δ^4 androstene 3 17 dione and testosterone were isolated (Savard *et al*, 1956*b*)

The finding of an 11β hydroxylase system previously thought to be limited to the adrenal, was unexpected and raises the question whether this gland studied by conventional pathological methods is derived from true testicular tissue or whether in spite of the pathological diagnosis adrenal tissue is contained in the tumour If the tumour is derived from testicular tissue and contains the 11β hydroxylating system (active in C_{19} steroids) does this mean that normal testicular tissue contains traces of this enzyme which is greatly increased in the tumour tissue? No ready answer is available but what is important is that by employing new biochemical techniques characteristics of the tumour were indicated which could not be inferred from pathological examination of the tissue In other words a field for study was uncovered

Worthy of comment is the fact that three 11β hydroxylated products were found when testosterone was used as the substrate, while no C_{21} 11β hydroxylated products could be detected when progesterone was incubated with the tumour tissue This latter experiment resulted in the detection of only the expected products of the androgen biosynthetic pathway In view of the report of Tomkins Michael and Curran (1957) concerning different 11β hydroxylating systems for different substrates the possibility must be considered that this inter

stitial cell tumour contains a C_{19} 11β hydroxylating system but no C_{21} 11β hydroxylating system

The production of steroids by what was originally diagnosed as a large undifferentiated testicular tumour in a 3 year old boy has been studied together with an analysis of urinary steroids (Baggett *et al* 1957) The true pathology of this abnormal tissue must still be considered to be in doubt since the original pathological diagnosis was questioned after the results of the biochemical studies became known to the pathologist The urine of this subject contained an elevated titre of both 17 ketosteroids and corticoids The urinary steroid pattern resembled that which might be expected from a patient with adrenal hyperactivity and included increased quantities of 11β hydroxyandrosterone dehydro*epi*androsterone aetiocholanolone urocortisone and urocortisol Incubation of the tumour tissue in the form of slices with carboxyl labelled acetate afforded [^{14}C]cortisol and presumptive evidence for 11β hydroxy Δ^4 androstene 3 17 dione Δ^4 androstene 3 17 dione and dehydro*epi*androsterone Here again is an instance of a routine pathological diagnosis which was upset by biochemical findings made possible by the use of newer techniques Obviously the biosynthetic pattern is not limited to that which would be expected in a normal gonad The presence of cortisol and dehydro*epi*androsterone is clearly an indication of the presence of adrenocortical tissue or severely modified testicular tissue if such is possible

An embryonal tumour of the testis was studied by Wotiz Davis and Lemon (1955) This tumour, when incubated with acetate led to the biosynthesis of testosterone and Δ^4 androstene 3 17 dione together with the oestrogens oestrone and oestradiol 17β No evidence for 11β hydroxylation could be found and the biosynthetic pathways indicated from the studies are those to be expected from normal testicular tissue

In an earlier study of steroids in the urine of a patient with chorionepithelioma of the testis with metastases relatively high concentrations of pregnanediol 17 ketosteroids and oestrogens were excreted (Laipply and Shipley 1945) No

studies of biosynthetic potential of the gland could be done at the time On the basis of available information it would appear that in this instance the biosynthesis of the steroid hormones proceeded in the expected manner except that all components of the pathway were present in increased amounts

Ovarian production of androgens is believed to proceed by a pathway analogous to that already described for the testis Experimental evidence for this idea comes from the work of Solomon, Wiele and Lieberman (1956–7), who demonstrated the conversion of progesterone to 17α hydroxyprogesterone and Δ^4 androstene 3 17 dione by bovine ovarian homogenates A 24 year old patient bearing an arrhenoblastoma with normal urinary 17 ketosteroids and corticoids showed facial and axillary hirsutism enlarged clitoris and amenorrhoea of six months' duration The tumour in the form of slices was incubated with [4 ^{14}C]progesterone and yielded the expected androgen intermediate 17α hydroxyprogesterone, and the androgens testosterone and Δ^4 androstene 3 17 dione The two possible 20 reduced products of progesterone, 20α hydroxy Δ^4 pregnen 3 one and 20β hydroxy Δ^4 pregnen 3 one were likewise demonstrated (Savard *et al*, 1957) No explanation for the virilization of the patient could be given on the basis of the reported findings since urinary titres were essentially normal and since the incubation studies yielded only qualitative information

Adrenal hyperactivity and tumours

The biosynthesis of corticoids both glycocorticoids and aldosterone has been elucidated in considerable detail and is represented in Fig 2 There seems to be little doubt that cholesterol is an important precursor of all the corticoids Whether an alternative pathway does exist involving a non cholesterol pathway is not clearly established Data from perfusion experiments (Stone and Hechter 1954) as well as adrenal incubation experiments (Heard *et al* 1956) point to this possibility

The finding of the 20β hydroxycholesterol in adrenal homo genates suggests that this steroid is an intermediate between cholesterol and the first C_{21} intermediate pregnenolone Whether it is necessary to postulate a second hydroxylation at $C_{(\ )}$ is not clear at this time but the studies of Solomon Levitan and Lieberman (1956–7) do indicate that if such a hydroxylation does take place it does so only after prior hydroxylation at $C_{(20)}$ This follows from the fact that no

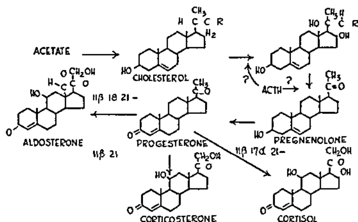


FIG 2 Biosynthesis of corticoids

monohydroxy $C_{(22)}$ (either 22α or 22β) could be detected in adrenal homogenates If a 20 22 dihydroxycholesterol is formed the conversion of this possible intermediate to preg nenolone should proceed by means of a reaction involving a proper desmolase Oxidation of pregnenolone to progesterone proceeds by means of the 3β ol dehydrogenase and progester one is hydroxylated at the various positions to cortisol corticosterone and aldosterone

The action of ACTH has been tentatively placed between cholesterol and pregnenolone As a working hypothesis this is reasonable but certain facts and ideas need clarification Heard and his co workers (1956) reported that ACTH stimu lates the 11β hydroxylation of cortexone to corticosterone

using hog adrenal sections If the concentration of reduced triphosphopyridine nucleotide (TPNH) in these adrenal sections is so low as to be rate limiting for the 11β hydroxylation step it is possible that ACTH could make available an increased quantity of TPNH which in turn would facilitate the reaction Such a mechanism could be visualized on the basis of the recent work of Haynes and Berthet (1957)

Adrenal androgen production most likely involves two pathways The first is similar to that already described for the gonads (Fig 1) and has been demonstrated by direct

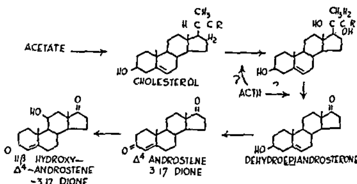


Fig 8 A pathway for adrenal androgen biosynthesis not involving a C_{21} intermediate

transformation experiments and the second, probably unique for the adrenals, would involve the direct metabolism of cholesterol to a C_{19} steroid such as dehydro*epi*androsterone without the need for a C_{21} intermediate (Fig 8) Evidence consistent with this latter biosynthetic pathway includes the appearance of androgens in human foetal adrenals before the appearance of significant amounts of corticoids (Bloch *Benirschke and Rosemberg 1956) the massive production of* C_{19} steroids in patients with adrenal cancer while only modest increments in C_{21} steroid production are evident the great increase in cortisol production in some patients with Cushing s syndrome without a proportionally increased production in

C_{19} steroids and the elevated corticoid production in certain types of stress at a time when 17 ketosteroid production may be decreased (Dorfman and Shipley 1956)

Adrenogenital syndrome

The incubation of slices derived from an adrenal gland of a 28 year old woman showing the signs and symptoms of the

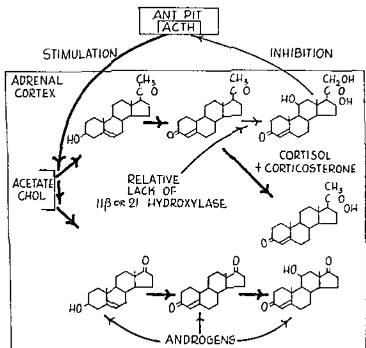


FIG 4 Biosynthesis of corticoids and androgens in patients with the adrenogenital syndrome

adrenogenital syndrome (Fig 4) was carried out in human serum with added glucose fumarate and [1 ^{14}C]acetate (Bloch Dorfman and Pincus 1957) The isolation of [^{14}C]de hydro*epi*androsterone provided the first demonstration of this steroid in adrenal tissue although indirect evidence for its presence had accumulated during the past twenty years The

additional androgens biosynthesized in this experiment include Δ^4 androstene 3 17 dione and 11β hydroxy Δ^4 androstene 3 17 dione Stimulation by ACTH produced the most striking (fivefold) increment in 11β hydroxy Δ^4 androstene 3 17 dione Increases in the concentration of the other two androgens were minimal The increased amount of the 11β hydroxylated product in the presence of ACTH is perhaps similar to the finding of Heard and his co workers (1956) that ACTH increases the yield of corticosterone from cortexone in an incubation system employing hog adrenal sections Both findings may be interpreted to mean that only a limited amount of TPNH is available and that the action of ACTH is to make adequate TPNH available (Haynes and Berthet 1957) That TPNH is essential for the hydroxylating reactions has been adequately demonstrated (Hayano *et al* 1956)

Initial studies on the adrenogenital syndrome clearly indicated that the effective concentration of 21 hydroxylase was relatively low (Dorfman and Shipley 1956) More recently this finding has been augmented by those of Eberlein and Bongiovanni (1956) who have clearly demonstrated that at least in some patients with the adrenogenital syndrome and hypertension there is a relative lack of an 11β hydroxylase which results in a high production of 11 deoxycortisol This conclusion is based on the finding of a high urinary excretion of aetiocholanolone and of the tetrahydro (3α 5β) reduction product (3α 17α,21 trihydroxypregnan 20 one) of 11 deoxycortisol

A patient who has been studied in detail by Ungar and Dorfman (1956) showed the signs and symptoms of Cushing s disease secondary to an adrenal cancer The patient was in the terminal stage of the disease that is the patient died within a few weeks of completion of the urine collections An unusually large amount of aetiocholanolone was found together with the tetrahydro derivative (3α 5β) of 11 deoxycortisol There is reason to believe that this latter steroid contributed to the bulk of the aetiocholanolone and originated from endogenously produced 11 deoxycortisol In the terminal

stages of the disease the adrenal of this patient apparently showed a severely reduced ability to 11β hydroxylate 11 deoxycortisol and in this respect the defect is similar to those described by Eberlein and Bongiovanni (1956) Touchstone and his co workers (1954) and Rosselet and his co workers (1954)

We have recently had the opportunity to study the bio synthetic potentials of an adrenal adenoma of a patient with

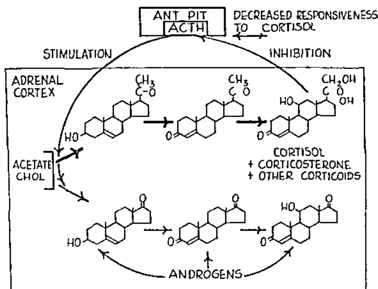


FIG 5 Biosynthesis of corticoids and androgens in Cushing s syndrome

the signs and symptoms of Cushing s syndrome (Goldstein and Dorfman unpublished results) Fig 5 illustrates a possible hypothesis of this disease The important features of the abnormal state may involve a decreased responsiveness of the anterior pituitary to the inhibitory action of cortisol (and/or other corticoids) so that a large amount of ACTH is produced in the face of high concentrations of corticoids Further in this clinical state the adrenal produces a disproportionately large amount of C_{21} steroids When the adenoma removed

from the 84 year old woman was incubated with [1 ^{14}C]acetate, ten steroids both C_{19} and C_{21}, were isolated containing ^{14}C Of particular interest is the finding of a much greater production of corticosterone and 11 dehydrocorticosterone than of cortisol and cortisone This was unexpected and points to the possibility that the disease may be maintained by a relatively high production of corticosterone and a relatively normal production of cortisol It further may explain why some patients with this disease may show normal blood and urinary levels of corticoids determined by a procedure such as that of Silber and Porter

Summary

Biosynthesis of steroid hormones in normal gonadal and adrenal tissues has been briefly reviewed Emphasis has been placed on the similarities and differences in steroid hormone production in states of hyperfunction of certain endocrine glands and functional tumours Examples are given wherein conventional pathological examination alone of endocrine tumours has been found inadequate to describe and classify the tissues properly However in these instances biochemical studies involving urinary steroid studies together with bio synthetic studies using tumour tissues have further clarified the nature of the pathological growths It is anticipated that studies such as these, involving both pathological and bio chemical techniques may lead to clearer interpretation of the nature and function of hyperfunctioning tissue and perhaps to a more precise classification of functional tumours

Acknowledgement

This work is supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research

REFERENCES

BAGGETT B ENGEL L L SAVARD K and DORFMAN R I (1956) *J biol Chem* 221 931

BAGGETT B ENGEL L L SAVARD K and DORFMAN R I (1957) *Fed Proc* 16 149

BLOCH E BENIRSCHKE K and ROSEMBERG E (1956) *Endocrinology* 58 626

BLOCH E DORFMAN R I and PINCUS G (1957) *J biol Chem* 224 737

DORFMAN R I and SHIPLEY R A (1956) Androgens Biochemistry Physiology and Clinical Significance New York John Wiley and Sons

EBERLEIN W R and BONGIOVANNI A M (1956) *Helv paediat acta* 2 105

HAYANO M SABA N DORFMAN R I and HECHTER O (1956) *Recent Progr Hormone Res* 12 79

HAYNES R C Jr and BERTHET L (1957) *J biol Chem* 225 115

HEARD R D H JELLINCK P H and O DONNELL V J (1955) *Endocrinology* 57 200

HEARD R D H BLIGH E G CANN M C JELLINCK P H O DONNELL V J RAO B G and WEBB J L (1956) *Recent Progr Hormone Res* 12 45

LAIPPLY T C and SHIPLEY R A (1945) *Amer J Path* 21 921

LYNN W S Jr (1956) *Fed Proc* 15 305

LYNN W S Jr and BROWN R (1956) *Biochim biophys Acta* 21 403

ROSSELET J P OVERLAND L JAILER J W and LIEBERMAN S (1954) *Helv chim acta* 37 1933

SAVARD K DORFMAN R I BAGGETT B and ENGEL L L (1956a) *Fed Proc* 15 346

SAVARD K DORFMAN R I BAGGETT B and ENGEL L L (1956b) *J clin Endocrin Metab* 16 1629

SAVARD K *DORFMAN R I GABRILOVE J L* and SOFFER L J (1957) Proc Endocrine Society 35th Meeting New York City p 133

SLAUNWHITE W R Jr and SAMUELS L T (1956) *J biol Chem* 220 341

SOLOMON S WIELE R V and LIEBERMAN S (1956) *J Amer chem Soc* 78 5453

SOLOMON S LEVITAN P and LIEBERMAN S (1956–7) *Rev canad Biol* 15 282

STONE D and HECHTER O (1954) *Arch Biochem Biophys* 51 457

TOMKINS G M MICHAEL P J and CURRAN J F (1957) *Biochim biophys Acta* 23 655

TOUCHSTONE J C RICHARDSON E M BULASCHENKO H LANDOLT I and DOHAN F C (1954) *J clin Endocrin Metab* 14 676

UNGAR F and DORFMAN R I (1956) *J clin Endocrin Metab* 16 920

VENNING E H HOFFMAN M M and BROWNE J S L (1942) *J biol Chem* 146 369

WEST C D DAMAST B L SARRO S D and PEARSON O H (1956) *J biol Chem* 218 409

WOTIZ H H DAVIS J W and LEMON H H (1955) *J biol Chem* 216 677

DISCUSSION

Finkelstein I would like to comment on the oestriol excretion of a dog reported by Laufer and Sulman, and later referred to by Dr Dorfman Actually it was I who performed the assay of the oestrogens of this dog s urine The assay was made fluorometrically In the oestriol fraction we obtained readings equal to about 50 µg oestriol per litre of urine I was very suspicious of this result so later on we chromatographed the extracts on paper but could not find any oestriol We also looked for oestrone and oestradiol but nothing was found at the level of 50 µg per litre of urine Then we assayed the urine by the classical Allen Doisy test and we went down to 200 mouse units per litre of urine but even on this low level we could not detect any oestrogen activity We found something on the paper but we could not identify it it was neither oestradiol nor oestriol On the other hand I believe Dr Sulman made his bioassays by application directly to the vagina and he found quite a lot of activity

Anyway we informed Drs Laufer and Sulman about our results and later on they published a correction on the oestrogen excretion (1957 *J clin Endocrin Metab* 17 164)

Horning Dr Laufer s dog had a Leydig cell tumour and it has been demonstrated that Leydig cells are capable of secreting oestrogen as well as androgens The recent cryptorchid experiments on rats carried out by Bielschowsky are very interesting He found that the cryptorchid testes developed hyperplasia in the Leydig cells sufficient to stimulate the mammary gland epithelium which suggests that the Leydig cells under certain conditions are capable of elaborating and secreting oestrogen

Finkelstein The problem of steroid excretion in the adrenogenital syndrome has attracted us for a considerable time Recently we reported the isolation of pregnane 3α 17α 20α triol 11 one (*pregnanetriolone*) from the urine of two females with pseudohermaphroditism due to congenital adrenal hyperplasia We discovered the presence of this steroid in the urine of those patients by means of a fluorescence reaction with 85 per cent phosphoric acid a technique which we originally developed for estimation of urinary oestrogens We have observed however that normal human urine also contains material which produces similar fluorescence although of low intensity In late pregnancy and in cases of adrenal tumours the fluorogenic material is excreted in concentrations much higher than normal as judged by the fluorescence reaction

We were curious to see whether in all these instances the fluorescence was due to pregnanetriolone and we tried to ascertain this by isolation procedures Although for these experiments quite large volumes of urine were employed we failed to isolate pregnanetriolone Then we tried to elaborate a reaction more specific for pregnanetriolone which would ultimately provide us with the answer as to whether the excretion of this compound is limited only to cases of adrenal hyperplasia We observed that if pregnanetriolone is put on filter paper in concentrations

DISCUSSION

Finkelstein I would like to comment on the oestriol excretion of a dog reported by Laufer and Sulman and later referred to by Dr Dorfman Actually it was I who performed the assay of the oestrogens of this dog s urine *The assay was made fluorometrically In the oestriol* fraction we obtained readings equal to about 50 µg oestriol per litre of urine I was very suspicious of this result so later on we chromatographed the extracts on paper but could not find any oestriol We also looked for oestrone and oestradiol but nothing was found at the level of 50 µg per litre of urine Then we assayed the urine by the classical Allen Doisy test and we went down to 200 mouse units per litre of urine but even on this low level we could not detect any oestrogen activity We found something on the paper but we could not identify it it was neither oestradiol nor oestriol On the other hand I believe Dr Sulman made his bioassays by application directly to the vagina and he found quite a lot of activity

Anyway we informed Drs Laufer and Sulman about our results and later on they published a correction on the oestrogen excretion (1957 *J clin Endocrin Metab* **17** 164)

Horning Dr Laufer s dog had a Leydig cell tumour and it has been demonstrated that Leydig cells are capable of secreting oestrogen as well as androgens The recent cryptorchid experiments on rats carried out by Bielschowsky are very interesting He found that the cryptorchid testes developed hyperplasia in the Leydig cells sufficient to stimulate the mammary gland epithelium which suggests that the Leydig cells under certain conditions are capable of elaborating and secreting oestrogen

Finkelstein The problem of steroid excretion in the adrenogenital syndrome has attracted us for a considerable time Recently we reported the isolation of pregnane 3α 17α 20α triol 11 one (pregnanetriolone) from the urine of two females with pseudohermaphroditism due to congenital adrenal hyperplasia We discovered the presence of this steroid in the urine of those patients by means of a fluorescence reaction with 85 per cent phosphoric acid a technique which we originally developed for estimation of urinary oestrogens We have observed however that normal human urine also contains material which produces similar fluorescence although of low intensity In late pregnancy and in cases of adrenal tumours the fluorogenic material is excreted in concentrations much higher than normal as judged by the fluorescence reaction

We were curious to see whether in all these instances the fluorescence was due to pregnanetriolone and we tried to ascertain this by isolation procedures Although for these experiments quite large volumes of urine were employed we failed to isolate pregnanetriolone Then we tried to elaborate a reaction more specific for pregnanetriolone which would ultimately provide us with the answer as to whether the excretion of this compound is limited only to cases of adrenal hyperplasia We observed that if pregnanetriolone is put on filter paper in concentrations

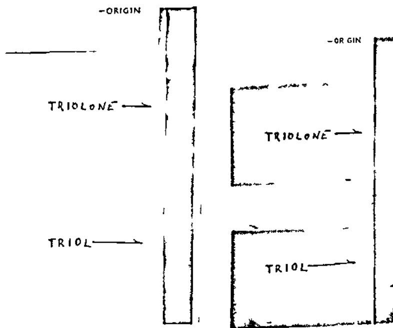


Fig 1 (a) Fig 1 (b)

Fig 1 (Finkelstein) Simultaneous estimation of pregnane 17α 20α tri and pregnane 3α 17α 20α triol 11 one on paper

System chloroform/formamide methanol (1 1)

Fig 1a in which pregnanetriol is visible was taken in daylight and Fig 1 in which pregnanetriolone is visible was photographed in u v light (3 660 with Wratten filter B in front of the camera

ff cing page

as low as 1–2 μg /sq cm the paper briefly moistened with 70 per cent phosphoric acid heated to 87 for 10 minutes then quickly cooled to room temperature and irradiated with u v light of maximal emission at 3 660 Å the compound produces a brilliant blue fluorescence Combined with paper chromatography this reaction has been found to be highly specific for pregnanetriolone (Finkelstein M and Goldberg S (1957) *J clin Endocrin Metab* 17, 1063) The same reaction was also applied to pregnane 3α 17α 20α triol which produces a pink fluorescence under these conditions and a test has been elaborated for simultaneous estimation of both compounds (Fig 1) (Finkelstein M and Cox R I (1957) *Proc Soc exp Biol N Y* 95 297) With this test we screened the urine of about 100 normal people and a urinary pool of 200 pregnancies but we could not detect pregnanetriolone above 40 μg /24 hr urine which under our conditions was the limit of sensitivity for the test In a few cases using more rigorous purification we put extracts of the entire *24 hour urine on paper and if only 2 μg of pregnanetriolone had been* present we should have been able to detect it But even at this low level no pregnanetriolone could be seen Moreover patients who for several days received injections of ACTH 150 mg /day did not excrete detectable amounts of pregnanetriolone although we had seen by the excretion of 17 ketosteroids and of pregnanetriol that the adrenals responded to the stimulation Furthermore in three cases of adrenal tumour we failed to detect any pregnanetriolone On the other hand we have had up to now 20 cases of female pseudohermaphrodites and boys with macrogenitosomia praecox both due to congenital adrenal hyperplasia and all of them invariably showed pregnanetriolone usually in concentrations above 1 mg /24 hr urine In a few cases of Cushing s syndrome with no tumour and tentatively diagnosed as adrenal hyperplasia although in some of them the adrenals were of normal appearance we have also detected pregnanetriolone in the urine but in concentrations of about 50–200 μg per 24 hours All this gives us reason to believe that the steroid excretion in adrenal hyperplasia is qualitatively different and that pregnanetriolone is specific for this condition We therefore hope that the analysis for pregnanetriolone may be useful for the differential diagnosis between adrenal tumour and adrenal hyperplasia but of course more cases of tumours must be analysed

That brings me to your scheme of the biosynthesis Dr Dorfman I wonder whether in the adrenogenital syndrome the β hydroxylation at $C_{(11)}$ doesn t occur before the hydroxylation at $C_{(21)}$ That means that if the hydroxylation at the 21 position is slow and the hydroxylation at $C_{(11)}$ proceeds at a normal rate then either $C_{(11)}$ hydroxy and/or $C_{(11)}$ keto $C_{(21)}$ deoxy compounds would mainly be formed In other words 21 deoxyhydrocortisone or 21 deoxycortisone would be produced It may be that these $C_{(21)}$ deoxy compounds oxygenated at $C_{(11)}$ are then hydroxylated at an even slower rate at the $C_{(21)}$ position which prevents normal production of cortisol

Dorfman That is not the usual experience with a 21 hydroxylating system from either mould or tissues Both 21 deoxycortisone and 21 deoxycortisol are excellent substrates for this enzyme system

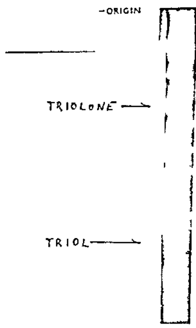


Fig 1 (a)

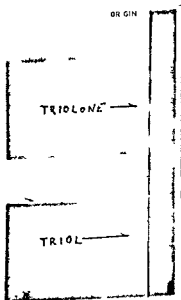


Fig 1 (b)

Fig 1 (Finkelstein) Simultaneous estimation of pregnane 3α 17α 20 triol and pregnane 3 17α 20α triol 11 one on paper

System chloroform/formamide methanol (1 1)

Fig 1a in which pregnanetriol is visible was taken in daylight and Fig 1b in which pregnanetriolone is visible was photographed in u v light (3 0(0 Å) with Wratten filter – B in front of the camera

[f cd g p ge 5

Dorfman That is the usual type of adrenogenital syndrome patient that is encountered and as a matter of fact we have never yet encountered a patient in whom the 11β hydroxylase is low except one with adrenal cancer in the late stages of the disease

Robinson My evidence supports yours but not some of the evidence that has been put forward at other conferences

Dorfman I think it is very clear that where Dr Bongiovanni found an excessive amount of both 17 and 21 hydroxylation 11β hydroxylation was certainly low

Robinson Yes of course Our material was quite characteristic and easy to isolate

Furth You may have over rated the pathologist s capacity for differentiating the functional cells in question I have convinced myself that on morphological grounds alone one cannot distinguish the Leydig cell tumour from luteomas or certain adrenocortical tumours which have a similar hormonal spectrum Pathologists are usually guided by clinical findings and the site of the tumour

Gray Were any of your tumours associated with abnormal gonadotrophin excretion and can this be tied up in any way with the enzymic abnormality?

Dorfman Neither of the patients that we showed with the tumour had increased gonadotrophin However there was a third patient whom I now take the opportunity to mention I am ashamed of this because I think that this presented the clues for the understanding of the mechanism of formation of androgens and I failed to recognize it at the time A patient with a chorionepithelioma of the testis excreted increased 17 ketosteroids increased pregnanediol (which should have been the tip off to us) and increased oestrogens That patient did have increased gonadotrophin (Laipply and Shipley 194_)

Finkelstein You mentioned a case of arrhenoblastoma with normal 17 ketosteroids We saw about the same in a case with adrenal tumour The question is why were these patients virilized?

Dorfman We have encountered a number of cases in which women have shown considerable virilism without corresponding values of high androgens and/or 17 ketosteroids Here you may invoke changes in tissue responsiveness or perhaps potentiators I cannot give a solid explanation

Finkelstein Another question is why in cases of adrenogenital syndrome does much more cortisone have to be administered to keep the syndrome down? The amount is much more than normal persons would require by endogenous production

Dorfman Perhaps if these facts are so this is due to hyperactivity of the hypothalamic anterior pituitary mechanism On the other hand I am not aware of good quantitative data that would support the idea of a relative lack of pituitary responsiveness

Pincus I think the reverse is very obvious if you overstimulate the adrenals and then withdraw the source of overstimulation there is a long latent period before they recover It may very well be that this hypothesis is correct but you can only tell by indirect means

Finkelstein Jailer has discussed the problem of there being a possible activator It could well be that hyperplastic adrenals produce a steroid or something which activates the pituitary

Crooke Have you any explanation of why there is a suppression of the 17 ketosteroid series and at the same time an increase of the cortisol series in one of the experiments you mentioned?

Dorfman At the moment I visualize this as a difference either in hydroxylating reactions or perhaps in the desmolase reactions When we have a cluster of molecules we have the possibility of these two hydroxylations at 17 and 20 or at 20 and 22 and perhaps a compound such as this may exist It depends on where the reaction takes place whether this will be a desmolase 1 or desmolase 2 Desmolase 2 would control the rate of formation of a C_{21} compound and desmolase 1 could conceivably control the formation of a C_{19} compound So the control could possibly be either an effective concentration of this splitting enzyme or it could be actually in the hydroxylation at 17 versus 22 20 being an anchor point for both types of reaction That is our working hypothesis now and we have a long road to go because what we are trying to do is to find and purify these specific enzymes in hormones

Huseby Pathologists would be most happy if you could diagnose the cell of origin of some of these testicular tumours by chemical means You mentioned that in normal testicular tissue you had not been able to demonstrate 11β hydroxylase Have you found 21 hydroxylase?

Dorfman We have not looked for 21 hydroxylase in normal testicular tissue

Huseby We have *in vitro* indications that 21 hydroxylase does occur in one of our induced testicular interstitial cell tumours in mice where it appears very unlikely that the tumour arose from an adrenal rest

Dorfman This is very exciting news

Huseby It is very disappointing too if you are trying to make a histological diagnosis chemically

Robinson We have made studies on several congenital hyperplasias and we discovered a considerable amount of 11 hydroxyandrosterone and 17 hydroxypregnanolone which may be relevant to the discussion on the competition of the hydroxylases In these cases we have had up to 20 mg a day of 17 hydroxypregnanolone and about 13 mg on an average of 11β hydroxyandrosterone

oestrous features in vaginal smears from castrate females invariably produced an unmistakable reaction in the weight of the seminal vesicles in the males

A cyclic production of oestrogens by granulosa cell tumours was observed by Crelin and Wolstenholme (1951) on the basis of the fact that the vagina in castrated tumour bearing mice showed features of three days oestrus or more alternating with five days dioestrus These authors indicate the possibility of a pituitary influence on the oestrogen production of tumours

Green (1955) also presumes that oestrogen production by ovarian tumours is dependent on the quantity of gonadotrophic hormone on the basis of the fact that in ovariectomized hosts the uterine weight can be increased both by administration of equine serum gonadotrophin and by chorionic gonadotrophin

Our experiments yielded no indication however of a cyclic production of oestrogenic hormone Daily control according to Thung Boot and Muhlbock (1956) of vaginal smears from tumour bearing animals showed the constant picture of the oestrous stage This result does not tend to suggest the probability of a pituitary influence on the production and secretion of oestrogenic hormones

It was also found that granulosa cell tumours were capable of growing and producing oestrogenic hormones following transplantation to hypophysectomized animals (Muhlbock, 1951 van Nie 1957) This is an observation which proves that oestrogenic hormone production without the influence of the pituitary is possible In these animals it was naturally possible only to demonstrate the oestrogenic effect on the vagina and the uterus and not that on the mammary glands as the pituitary hormones required for the growth of this organ were lacking Comparison of the extent of hormone production in hypophysectomized animals with that in normal animals is difficult as tumour growth is slower in the first category Table I shows however that the size of the tumour in hypophysectomized animals in which an oestrogenic effect was first observed does not considerably differ from the tumour

THE PRODUCTION OF OESTROGENIC HOR MONES BY GRANULOSA CELL TUMOURS IN MICE

O Muhlbock, R van Nie and L Bosch
The Netherlands Cancer Institute Amsterdam

The production of oestrogenic hormones by granulosa cell tumours is a long established phenomenon Gardner, Strong and Smith (1936) were the first to mention hormonal activity of bilateral ovarian granulosa cell tumours in mice—an activity which was more exhaustively described by Strong Gardner and Hill (1937) as follows vaginal smears from female animals submitted to implantation of a spontaneous ovarian tumour showed the features of continuous oestrus, which were maintained after castration of the host but changed into the features of dioestrus after surgical extirpa tion of the tumour The male hosts showed stimulation of mammary growth Bali and Furth (1949) likewise observed an oestrogenic influence on the vagina, uterus ovaries, sub maxillary glands testes and seminal vesicles in hosts carrying transplantable granulosa cell tumours The percentage of animals showing this effect was dependent on the size of the tumours

Oestrogen producing ovarian tumours have also been re ported in rats (Iglesias Sternberg and Segaloff, 1950 Peck ham and Greene 1952)

In our laboratory, too we were able to confirm oestrogen production by transplanted ovarian tumours in mice The following two criteria were used in this confirmation, viz the occurrence of oestrous features in vaginal smears from castrate female hosts and the decrease in seminal vesicles and in testicular weight in male hosts Both reactions were deter mined in the majority of cases Tumours which gave rise to

The oestrogenic hormone production by different granulosa cell tumours varies considerably This fact was demonstrated very convincingly by studying the metabolic activity of the uterus in ovariectomized mice at successive periods after transplantation of the tumour Isolated uterine tissue segments were incubated at 37° with [1 ^{14}C]acetate and the *in vitro* incorporation of ^{14}C into proteins cholesterol and fatty acids was measured In the case of one tumour (5441) no oestrogenic activity could be detected by means of vaginal smears The *in vitro* activity of the uterine metabolism of the ovariectomized host clearly showed that this tumour produced oestrogenic compounds These metabolic studies also revealed the hormone secretion at a much earlier stage than that at which any oestrogenic activity could be demonstrated by vaginal smears (Bosch and Emmelot 1956)

The question then arises as to whether the hormones produced by these tumours are identical with those produced by the normal ovaries namely oestrone oestradiol and oestriol In order to settle this question an attempt was made at identification of the oestrogenic hormones produced by granulosa cell tumours (Bosch 1955) The first step was an investigation into the possibility of demonstrating oestrogenic substances in extracts of the tumours The oestrogenic potency of the extracts was tested by the vaginal smear technique The oestrogen content of the tumours studied corresponded with an amount of oestrone which varied from 10^{-4}–3 μg per gram of lyophilized tissue The tumour which showed the highest hormone content (19957) was selected for an attempt to identify the oestrogens chemically The biologically active extracts retained their activity after very elaborate purifications which specifically aimed at the isolation of phenolic steroids In a single case paper chromatography revealed the presence of oestrone and oestradiol The conclusion can therefore be drawn that it is highly probable that the oestrogens produced in ovarian tumours are the same as in the normal ovary In order to gain some information about steroid synthesis in these ovarian tumours Bosch (1955)

Table I

TUMOUR WEIGHT AT FIRST APPEARANCE OF OESTROUS VAGINAL SMEARS IN OVARIECTOMIZED MICE (SUBCUTANEOUSLY GRAFTED)

	No of Mice	*Tumour Weight mg*	*Days after Grafting*
With Hypophysis	8	130–372 (236)	14
Without Hypophysis	5	145–399 (246)	20

size in normal animals It should be taken into account, meanwhile, that the threshold value of the effective oestrogen dose in hypophysectomized animals is decreased (Muhlbock and van Maurik 1951)

In these ovarian tumours too the phenomenon of progression is seen i e the tumours no longer produce hormones under certain conditions which cannot always be defined The morphological aspects of these tumours sometimes show changes, although a tumour may also cease to produce hormones without a change in the morphological picture If the morphology changes then this change is exclusively towards increased anaplasia The tumours then resemble sarcomata The tumours no longer produce oestrogens following a sarcomatoid transformation This transformation is irreversible, in no case has a transformation in reverse been observed The factors to which this transformation should be attributed are still unknown Our experience with mammary tumours suggests that a genetic factor must be involved There is some reason, moreover to presume that this increase in autonomy is promoted if the tumour cells are under unfavourable growth conditions as a result of which their growth is retarded Once the transformation has been completed however the rate of growth is higher than that in the original tumour (Rijssel *et al*, 1954)

as much as the above The ability of the liver to break down oestrogens probably does not decrease until after some considerable time In order to verify the correctness of the presumption of a reduced oestrogen breakdown in the liver hepatic sections from mice with and without ovarian tumours were incubated after the addition of oestrone The results tend to confirm that the enzymic system involved in the breakdown of oestrogens in the livers of tumour bearing mice

Table II

TUMOUR WEIGHT AT FIRST APPEARANCE OF OESTROUS VAGINAL SMEARS IN OVARIECTOMIZED MICE

Tumour Site	*No of Mice*	*Tumour Weight g*	*Days after Grafting*	*Oestrous Symptoms*
Subcutaneously	8	0 130–0 [illegible]72 (0 236)	14	+
In Kidney	11	0 002–0 113 (0 0311)	28	+
In Spleen	7	1 0–3 3 (2 2)	42	—

is inferior in activity to that in the livers of normal animals (Bosch 1955)

Considerable differences in hormone production have been found between various granulosa cell tumours In addition the same tumour may show a change in hormone production associated with transplantation Mention has been made of the sarcomatoid transformation of granulosa cell tumours which is associated with an arrest of hormone production

The production of oestrogenic hormones can also be reduced however without a change in the morphological aspect Granulosa cell tumour 5441, for example caused a positive oestrous reaction in castrate animals from the 23rd to the 32nd transfer passage at an average tumour weight of 0 24 g In the 59th transfer passage a positive oestrous reaction was

studied the *in vitro* biosynthesis of steroids by incubating surviving tumour slices with [1 ^{14}C]acetate After addition of carrier oestrone and oestradiol to the incubation mixture at the end of the experiments no radioactivity could be detected in the oestrogens isolated Probably the synthesis in these tumours is so slow that it cannot be measured by this method

The method most frequently used to induce ovarian tumours in mice consists of implantation of the ovary into the spleen in a castrate animal The oestrogenic hormones produced in the ovary are then broken down in the liver, so that the hormonal conditions in the animal are the same as those found in a castrate In the case of induction of granulosa cell tumours in the spleen however, there is invariably the striking feature of marked uterine growth and of a demonstrable oestrogenic effect in the vagina Since chemical investigations have shown the probability that in the tumours the same hormones are produced as in the normal ovary it seems justifiable to presume that the ability of the liver to break down *oestrogenic hormones is considerably reduced in the presence* of tumours Oestrogenic hormones consequently enter the circulation in this manner and cause the symptoms characteristic of these hormones The following test shows that the liver is capable of breaking down the oestrogenic hormones produced in the ovarian tumours

Tumour tissue was transplanted into spayed female mice, subcutaneously in the first group into the kidney in the second group and into the spleen in the third The tumour weight was determined in the three groups as soon as the first oestrous manifestations occurred after transplantation Table II shows that subcutaneous tumours with an average weight of 0 236 g produced a quantity of oestrogen sufficient to cause a positive vaginal oestrous reaction In the group with transplantation into the kidney this reaction was positive when the tumour weight was still considerably less The reaction in the animals with a tumour transplanted into the spleen remained negative all oestrogens were broken down in the liver even when the tumours reached a weight ten times

Line II showed oestrogen production from passage 6 on wards The biological differences between the tumours of these two lines were not associated with a histological difference The microscopic sections of both lines were not distinguishable both were of the luteoma type in which the degree of lutein ization can vary Production of other hormones especially of progesterone could not be demonstrated Mention should be made of the observation that the hosts of the tumours in line I repeatedly showed metastases which was never the case in

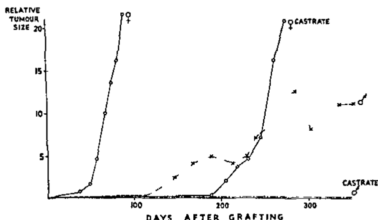


FIG 2 Influence of gonads on the growth of transplanted luteoma 7331

hosts of line II It might be possible that the slower growth rate in line I is of importance in that respect Another feature is that the tumours of line I grow better in females than in males while the tumours of line II showed the opposite de pendence (van Nie 1957) The hormone dependence of line I is shown in Fig 2 In females the growth of tumours of line I is most satisfactory After seven months growth will occur in ovariectomized females also In males growth is very slow and no growth was seen in castrated males even after a year

It can be presumed that oestrogens promote the growth of these tumours As could be shown in the following experiment

no longer demonstrable, not even if the tumours were considerably larger, weighing up to 5 g

The reverse—oestrogen production confined to later transfer passages—could also be observed One transplanted tumour caused striking cachexia in the hosts (Fig 1) No oestrogen production was observed This behaviour of the tumour was seen until the 50th transfer passage, after which it changed Cachexia was no longer seen in the 77th passage in which the tumour bearing host showed the typical signs of oestrogen

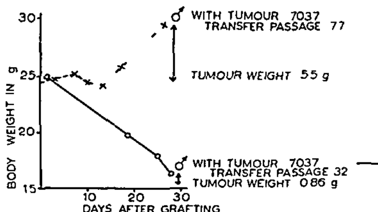


FIG 1 Decrease in cachexia caused by tumour 7037

production An explanation of these changes is difficult In regard to the oestrogen production it could be that the size of the tumour is decisive In the cachectic animals the tumours were small whereas in the non cachectic animals the tumours were much larger

The fact that changes in the environment can influence the growth of these tumours can be demonstrated in the case of an ovarian tumour, a luteoma which developed spontaneously in a DBA female The first passage was grafted in female mice After that one line was exclusively grafted in females whereas line II was exclusively grafted in males The tumours of line I never showed any oestrogenic hormone production

That this high dose of oestrone has an inhibiting effect on the growth of this tumour is shown in Fig 4 In curve II it can be demonstrated that the tumour regresses when the high dose of oestrone is given This regression is only transient After 30 weeks the tumour resumes its growth in spite of the continued application of this high dose of oestrone If this tumour is transplanted then growth only occurs in the presence of a high dose of oestrone The hormone dependency of the tumour therefore can be changed by the hormonal environment in which the tumour is growing (van Nie 1957)

The following is a survey of the ovarian tumours used in this investigation

(1) 54 transplantable ovarian tumours were obtained by transplantation of 154 ovarian tumours
(2) Of these 54 transplantable tumours
 9 developed spontaneously
 10 by X ray induction
 33 by intrasplenic grafting
 2 by subcutaneous implantation of an ovary
(3) 52 were of the granulosa cell type
 2 were of the luteoma type
(4) In 7 sarcomatoid transformation occurred in one a haemangioma was formed

The biological behaviour of 19 granulosa cell tumours is compared in Table III Of these 19 different tumours 7 produced a marked 10 a weak and 2 no oestrogen effect in the hosts The large majority of the granulosa cell tumours tested therefore produced oestrogens to some degree From these 19 tumours it was also determined whether an increased blood volume and sinusoid dilations in the liver and the adrenals were demonstrable Hypervolaemic manifestations were found in 10 of these tumours This shows that tumours with a high oestrogen production do not always show hypervolaemia On the other hand however no tumours were found which showed hypervolaemia while producing no oestrogenic hormones

A comparison between oestrogen production and hormone

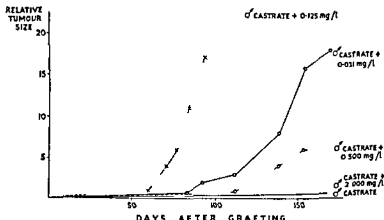


FIG 3 Influence of different doses of oestrone administered in drinking water on transplanted ovarian tumour 7331

(Fig 3) the growth of these tumours is, however largely dependent on the dose administered A maximal effect was seen when the animals were given 0 125 mg oestrone per litre of drinking water A lower dose had less effect It is remarkable however that higher doses have no growth stimulating effect and with the highest dose of 2 mg oestrone per litre of drinking water tumour growth was completely suppressed

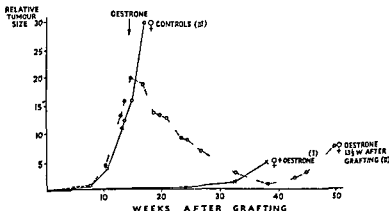


FIG 4 Influence of 2 000 mg oestrone per litre of drinking water on the growth of the transplanted ovarian tumour 7331

Tumours grafted in males show more satisfactory growth than those grafted in females and castrates That was demonstrated in 16 out of 20 granulosa cell tumours investigated Tumour growth is inhibited by orchidectomy of tumour bearing hosts Administration of male sex hormones to castrates can promote tumour growth in these animals This effect is dependent on the dose administered Although granulosa cell tumours as a rule show unsatisfactory growth in female mice oestrone can nevertheless promote tumour growth However relatively large doses of oestrone are required for it The growth of granulosa cell tumours is also stimulated by other steroid hormones such as progesterone and cortexone This growth promoting effect is not dependent on the steroid structure of the hormones a similar effect can be obtained by means of stilboestrol (3 4 di *p* hydroxyphenylhex 3 ene)

The hormone production and the hormone dependence of transplanted ovarian tumours raise the question of what influence the steroid hormones have on the development of these tumours (Tables IV V VI) Generally it is assumed that the inciting factor in the genesis of these tumours is the follicle stimulating hormone (FSH) from the hypophysis Under experimental conditions after grafting of an ovary into the spleen a relative overproduction of the gonadotrophic hormone acts on the ovary and induces granulosa cell tumours

In most transplanted granulosa cell tumours testosterone has a growth promoting effect Treatment with testosterone of an animal with an intrasplenic graft of an ovary completely inhibits the development of an ovarian tumour (Table IV) The same is true if the ovary is implanted into the spleen of an intact male If treatment with testosterone of a castrated animal with an intrasplenic graft of an ovary is started 10 months after the grafting—at a time when ovarian tumours are already palpable—no accelerating influence of testosterone on the growth of the tumour can be observed

It may be contended that an accelerating effect of testosterone is masked by an inhibiting effect of testosterone on the gonadotrophic hormone production of the hypophysis

Table III

BIOLOGICAL CHARACTERS OF 19 DIFFERENT TRANSPLANTED GRANULOSA CELL TUMOURS

Tumour	*Oestrogenic effect*	*Hypervolaemic effect*	*Hormone dependency*
5438	+	+	++
5441	++	+	++
7037	–	–	+
18850	+	+	+
19957	++	–	–
19958	++	–	++
19959	+	+	++
21605	–	–	++
23615	+	+	+
24201	++	+	–
24203	+	+	++
24260	++	–	+
24262	+	–	–
26 67	+	–	+
28268	++	+	+
29709	+	+	–
33871	+	–	+
48777	+	–	+
56339	++	+	++

dependence fails to show a complete correlation There are tumours which produce oestrogenic hormones but show no hormone dependence and *vice versa*

The hormone dependence of the grafted granulosa cell tumours can be summarized as follows (van Nie 1957)

Tumours grafted in males show more satisfactory growth than those grafted in females and castrates That was demonstrated in 16 out of 20 granulosa cell tumours investigated Tumour growth is inhibited by orchidectomy of tumour bearing hosts Administration of male sex hormones to castrates can promote tumour growth in these animals This effect is dependent on the dose administered Although granulosa cell tumours as a rule show unsatisfactory growth in female mice oestrone can nevertheless promote tumour growth However relatively large doses of oestrone are required for it The growth of granulosa cell tumours is also stimulated by other steroid hormones such as progesterone and cortexone This growth promoting effect is not dependent on the steroid structure of the hormones a similar effect can be obtained by means of stilboestrol (3 4 di *p* hydroxyphenylhex 3 ene)

The hormone production and the hormone dependence of transplanted ovarian tumours raise the question of what influence the steroid hormones have on the development of these tumours (Tables IV V VI) Generally it is assumed that the inciting factor in the genesis of these tumours is the follicle stimulating hormone (FSH) from the hypophysis Under experimental conditions after grafting of an ovary into the spleen a relative overproduction of the gonadotrophic hormone acts on the ovary and induces granulosa cell tumours

In most transplanted granulosa cell tumours testosterone has a growth promoting effect Treatment with testosterone of an animal with an intrasplenic graft of an ovary completely inhibits the development of an ovarian tumour (Table IV) The same is true if the ovary is implanted into the spleen of an intact male If treatment with testosterone of a castrated animal with an intrasplenic graft of an ovary is started 10 months after the grafting—at a time when ovarian tumours are already palpable—no accelerating influence of testosterone on the growth of the tumour can be observed

It may be contended that an accelerating effect of testosterone is masked by an inhibiting effect of testosterone on the gonadotrophic hormone production of the hypophysis

Experiments were therefore done with mice in parabiotic union A castrated animal with an ovary transplanted into the spleen was united with a castrated partner The animal bearing the intrasplenic graft was treated with testosterone (2 mg as a pellet 3 times a week) After an average time of

Table IV

INFLUENCE OF TESTOSTERONE ON THE GENESIS OF OVARIAN TUMOURS AFTER INTRASPLENIC GRAFTING OF AN OVARY

	WEIGHT OF THE OVARIAN TUMOUR IN mg	AGE IN DAYS
	2180	380
TESTOSTERONE	0	290
MA ES	0	485
AFTE 10 MO S ES OSTERONE	1760	380
TESTOSTERONE	180	249
	259	197
TESTOSTERONE	546	210
	484	311

249 days only small ovarian tumours were seen Again it may be contended that testosterone treatment inhibits the gonado trophic potency of the hypophysis in both partners although it is known that the crossing of testosterone to the untreated partner is almost nil In the next series of experiments a castrated animal with an intrasplenic ovarian graft was united with two castrated partners in the manner indicated in Table IV With this arrangement the castrated partner at

the outside cannot be influenced by the testosterone given because testosterone which may cross over from the first to the second partner is completely destroyed in the latter The third partner produces therefore uninhibited gonadotrophic hormone which can cross the two parabiotic unions (Muhlbock 1953) Testosterone has only a slight enhancing effect on the development of the ovarian tumour The same effect is seen

Table V

Influence of oestrone on the genesis of ovarian tumours after intrasplenic grafting of an ovary

	Weight of the ovarian tumour in mg	Age in days
	2180	380
	0	466
After 10 months oestrone	914	380
	259	197
Oestrone	240	251

in the animal with the intrasplenic graft in an intact male It must be added that the evaluation of these experiments is difficult because as has been already stated by Gardner (1955) the progression of the ovarian tumours in intrasplenic grafts is erratic and variable Nevertheless it can be said that the influence of testosterone on the development of primary ovarian tumours in intrasplenic grafts is not very striking

The same series of experiments was done with oestrone treatment (Table V) Oestrone given to an animal with an

intrasplenic graft completely inhibits the development of an ovarian tumour The same is true if this animal is only unilaterally ovariectomized When oestrone treatment is begun 10 months after the grafting of the ovary and continued for 3 months then significantly smaller ovarian tumours develop in the treated animal as compared with the untreated control

Table VI

Influence of progesterone on the genesis of ovarian tumours after intrasplenic grafting of an ovary

	Weight of the ovarian tumour in mg	Age in days
	2180	380
Progesterone	400	316
	259	197
Progesterone	532	321

In experiments with parabiotic triplet mice oestrone given in 2 mg pellets of 25 per cent oestrone plus 75 per cent cholesterol once every two weeks has no influence on the weight of the tumours In the evaluation of these experiments it must be considered that possibly the production of oestrogens in the tumour has already reached a high enough level so that additional treatment with oestrone has no influence

The third set of experiments was done in the same manner with progesterone (Table VI) Progesterone has a growth accelerating influence on transplanted granulosa cell tumours Its inhibitory effect on the gonadotrophic hormone production is weak Treatment with progesterone pellets (2 mg 3 times

weekly) has in single animals a retarding effect on the development of ovarian tumours Progesterone given to mice in parabiotic union with two castrated partners has again no striking influence on the development of these tumours

REFERENCES

BALI T and FURTH J (1949) *Cancer Res* 9 449
BOSCH L (1955) Ph D Thesis Delft
BOSCH L and EMMELOT P (1956) *Arch Gynaek* 188 172
CRELIN E S and WOLSTENHOLME J T (1951) *Cancer Res* 11 212
GARDNER W U (1955) *Cancer Res* 15 109
GARDNER W U STRONG L C and SMITH G M (1936) *Amer J Cancer* 26 541
GREEN J A (1955) *Proc Amer Ass Cancer Res* 2 20
IGLESIAS R STERNBERG W and SEGALOFF A (1950) *Cancer Res* 10 668
MUHLBOCK O (1951) *Ned Tijdschr Geneesk* 95 3672
MUHLBOCK O (1953) *Acta endocr Copenhagen* 12 105
MUHLBOCK O and VAN MAURIK G (1951) *Acta physiol pharm néerl* 2 80
VAN NIE R (1957) Ph D Thesis Utrecht
PECKHAM B M and GREENE R R (1952) *Cancer Res* 12 25
VAN RIJSSEL TH G VAN NIE R MUHLBOCK O and DE BRUYN W M (1954) *4e Jaarb v Kankeronderz en Kankerbestr in Ned* 17
STRONG L C GARDNER W U and HILL R T (1937) *Endocrinology* 21 269
THUNG P J BOOT L M and MUHLBOCK O (1956) *Acta endocr Copenhagen* 23 8

DISCUSSION

Furth Since both oestrogen and progesterone stimulate the uterus how can one distinguish by mere radioactivity measurements whether the effects were due to oestrogens or progesterone?

Muhlbock From these experiments I could not distinguish but in other experiments we have never found any evidence that these tumours produce progesterone

Furth In principle though this is not an oestrogen assay but an assay of rate of uterine growth

Pincus Progesterone is relatively ineffective we have to use much larger doses of it than of oestrogen to obtain growth of the uterus

Furth You mentioned that even though you could demonstrate oestrogen by extraction *in vivo* assays were unsuccessful I wonder whether some co-factor was missing

Dorfman The important thing is that at best the radiochemical yield is exceedingly low One factor is the amount of radioactivity one employs There is a very long pathway from acetate to cholesterol to

progesterone to androgen to oestrogen and I believe most experiments have been negative only because insufficient amounts of ^{14}C were used Successful experiments demonstrating biosynthesis of oestrogens from acetate have been reported (Rabinowitz J L and Dowben R M (1955) *Biochim biophys Acta* 16 96)

Gardner In those tumours in which one finds for example two or three different types of cells do you suppose that these cells existed in the original tumour transplant and nothing was really changed but that different types were selected which existed initially? Are we really selecting instead of modifying?

Furth In our experience the original ovarian tumours are usually complex In the course of the first few passages both selection and differentiation occur Selection is the usual event Once a transplantable granulosa type of tumour is well established it remains of the granulosa type Similarly the luteomas and tubular adenomas remain true to type In the course of the first passages one notes in sections a suggestive transformation of adenomas to granulosa cells and of granulosa cells to luteomas It is known that the fibroadenoma of the rat can be changed by hormones Androgens stimulate connective tissue causing a sar coma like growth and oestrogens encourage the epithelial type of growth (Heiman J (1943) *Cancer Res* 3 65) (unconfirmed)

Muhlbock In the case I showed you the tumour always had a uniform morphological aspect of the luteoma type The tumour was in the 20th transfer passage at the start of the experiment With high doses of oestrone it showed a regression and afterwards it was dependent on these high doses Therefore we probably cannot speak of selection in this case

Furth The situation is analogous to that of thyrotrophic tumours At first they are inhibited by thyroid hormone but by keeping them ex posed to this hormone they acquire autonomy Later they may even grow faster in the presence of thyroid hormone This seems to be a general biological phenomenon commonly encountered by anti carcino gens The opinion whether environment selects a natural mutant or induces an adaptive modification or mutation is being debated

Gardner How long had the ovarian tumour that was pituitary inde pendent been transplanted? Was this independence true of all the ovarian tumours or how many did you test in that particular way? I have had some experiences that were different

Muhlbock This particular tumour I showed you has been transplanted for a long time indeed we observed this fact as long as five years ago and we repeated the experiment last year We have not tested all these tumours only about four or five of them Of course we selected them to show whether they were dependent on the hypophysis or not

Gardner Had the one that failed to respond to the animal s own gona dotrophin been transplanted for a long time?

Muhlbock Yes it had

Gardner That may make quite a difference We have had some tumours that will not grow in hypophysectomized animals and others that will I think it depends on the tumour that one selects and possibly

on the period of successive transplantation that that tumour has experienced I think one can select tumours that will show any of a number of different types of dependencies or responses

Muhlbock The point is that if they grow they produce oestrogenic hormones so that it shows that the oestrogenic hormone production is independent of the hypophysis

Horning Is the latent period of tumours after grafting gradually decreased during successive serial generation?

Muhlbock It is in most cases This particular luteoma I showed here was at first transplanted once a year but now after five or six years it is done every three months

Horning The first lot of kidney tumours I got to grow as subcutaneous transplants remained latent for nine months in the first generation They are now in their 20th generation as serial transplants and palpable tumours arise after about three months and sometimes before

Huseby In the experiments you made on the intrasplenic tumours in animals with large uteri what was the situation as far as the adrenals were concerned? Are you certain that oestrogen was being produced by the tumours rather than by hyperplastic adrenals?

Muhlbock I cannot say what the situation was in this particular instance but at any event we do not find hyperplasia of the adrenals in these animals

Huseby In experiments that we did several years ago we found that ovaries transplanted into the spleen exerted no inhibiting effect upon the development of postcastrational adrenal hyperplasia For instance castrate JK females possessing intrasplenic ovaries would come into positive oestrus within four months as a result of adrenal hyperplasia and still many months later they would develop intrasplenic ovarian tumours We tested this in several strains and found that in none did the ovary in the spleen inhibit the development of adrenal hyperplasia

Muhlbock The F_1 hybrids that we used did not show so much hyperplasia

Finkelstein When you isolated oestrone and oestradiol by paper chromatography was all the activity due to these hormones?

Muhlbock I do not know There was certainly no oestriol only oestradiol and oestrone

Furth In our experience animals that had an adrenocortical nodule were less likely to develop intrasplenic tumours This suggests that the adrenal cortex secretes oestrogens which inhibit ovarian tumour development This dependency vanishes when autonomy is acquired The time sequence probably varies with different strains

Huseby In the experiments that we did with several strains we got both intrasplenic ovarian tumours and adrenocortical hyperplasia One did not protect against the other at all

Furth What I mean is that this is a matter of quantity and time of acquisition of autonomy and its degree

Muhlbock But in our cases we have seen hypertrophy of the uterus

Gardner We might find in these tumours mechanisms for the inactivation of hormones added to the environment We may not necessarily

be concerned with the effects of a modification of hormones that we add to the environment but with metabolic derivatives of these hormones It is not particularly surprising for example that an ovarian tumour will grow in a hypophysectomized animal particularly if it is producing a lot of oestrogens because to some extent oestrogens would modify or reduce the gonadotrophin production by the pituitary were it present It would be quite natural if some of them did this Whether these injected hormones are modified by a tumour is something that investigations of the type that Dr Dorfman is doing will show and that might prove quite interesting

GENERAL DISCUSSION

Woolley I think Prof Gardner and Dr Allen long ago had a partial answer to the suggestion that tumours modify endocrine secretory activity It might be a generalized inanition type of phenomenon Animals with breast tumours ceased to have oestrous cycles as tumour size increased

Gardner These ovarian tumours stop secreting oestrogen when the diet is reduced to about two thirds This affects some of the other tumours as well

Leathem The suggestion is that protein depletion interferes with pituitary synthesis Does that imply that amino acids are necessary for the production of gonadotrophins?

Woolley I think that might be true We observed that rapid growing androgen producing adrenocortical tumours failed to produce hormones as abundantly as the slow growing tumours of this type Perhaps utilization of basic materials might be involved

Furth Didn t Roy Hertz show that certain vitamins tremendously enhance the oestrogen effect?

Dorfman I think that one might say that in the absence of certain vitamins particularly folic acid the response to oestrogens is reduced I do not think you get super effects

Furth I thought Hertz found a tremendous enlargement in the oviduct in his chicks

Dorfman Yes this was due to the very large concentrations of oestrogens that he used What he showed was that there was a remarkably reduced oestrogen response in chicks deprived of folic acid

Huseby I am wondering a bit about this question of the loss of body weight and the inanition secondary to tumour growth and its effect upon hormone production I asked Dr Muhlbock about the granulosa cell tumour he described that produces oestrogen until it grows to a large size and then hormone production declines In general this would appear to fit in with the ideas expressed by Dr Woolley However Dr Muhlbock also said that the same sequence of events occurs in hypophysectomized animals Thus it appears that the problem is more complex because in this latter situation the inanition effect of the enlarging tumour could not be brought about by a suppression of the production of gonadotrophins by the pituitary

Leathem It would be interesting to know whether the granulosa

be concerned with the effects of a modification of hormones that we add to the environment but with metabolic derivatives of these hormones *It is not particularly surprising for example* that an ovarian tumour will grow in a hypophysectomized animal particularly if it is producing a lot of oestrogens because to some extent oestrogens would modify or reduce the gonadotrophin production by the pituitary were it present It would be quite natural if some of them did this Whether these injected hormones are modified by a tumour is something that investigations of the type that Dr Dorfman is doing will show and that might prove quite interesting

GENERAL DISCUSSION

Woolley I think Prof Gardner and Dr Allen long ago had a partial answer to the suggestion that tumours modify endocrine secretory activity It might be a generalized inanition type of phenomenon Animals with breast tumours ceased to have oestrous cycles as tumour size increased

Gardner These ovarian tumours stop secreting oestrogen when the diet is reduced to about two thirds This affects some of the other tumours as well

Leathem The suggestion is that protein depletion interferes with pituitary synthesis Does that imply that amino acids are necessary for the production of gonadotrophins?

Woolley I think that might be true We observed that rapid growing androgen producing adrenocortical tumours failed to produce hormones as abundantly as the slow growing tumours of this type Perhaps utilization of basic materials might be involved

Furth Didn t Roy Hertz show that certain vitamins tremendously enhance the oestrogen effect?

Dorfman I think that one might say that in the absence of certain vitamins particularly folic acid the response to oestrogens is reduced I do not think you get super effects

Furth I thought Hertz found a tremendous enlargement in the oviduct in his chicks

Dorfman Yes this was due to the very large concentrations of oestrogens that he used What he showed was that there was a remarkably reduced oestrogen response in chicks deprived of folic acid

Huseby I am wondering a bit about this question of the loss of body weight and the inanition secondary to tumour growth and its effect upon hormone production I asked Dr Muhlbock about the granulosa cell tumour he described that produces oestrogen until it grows to a large size and then hormone production declines In general this would appear to fit in with the ideas expressed by Dr Woolley However Dr Muhlbock also said that the same sequence of events occurs in hypophysectomized animals Thus it appears that the problem is more complex because in this latter situation the inanition effect of the enlarging tumour could not be brought about by a suppression of the production of gonadotrophins by the pituitary

Leathem It would be interesting to know whether the granulosa

be concerned with the effects of a modification of hormones that we add to the environment but with metabolic derivatives of these hormones It is not particularly surprising for example that an ovarian tumour will grow in a hypophysectomized animal particularly if it is producing a lot of oestrogens because to some extent oestrogens would modify or reduce the gonadotrophin production by the pituitary were it present It would be quite natural if some of them did this Whether these injected hormones are modified by a tumour is something that investigations of the type that Dr Dorfman is doing will show and that might prove quite interesting

does not distinguish between propylthiouracil and iodine deficiency Propylthiouracil is really simulating iodine deficiency as regards the pituitary and the pituitary is putting out more TSH

Furth It is true that the pituitary cannot distinguish between propylthiouracil and iodine deficiency and goitrogenic baby food (cereal) It responds with the same changes as following surgical thyroidectomy

The difference between destroying normal tissue and thyroid tumour by radioiodine is due to the relative concentration of ionizing energy per cell There is a tremendous uptake but there is a tremendous tumour mass and the ionizing irradiation per cell is probably less than that given to normal cells

Tata It has been calculated that in some cases the radiation delivered per square centimetre of the tumour tissue can approach the level of radiation which destroys normal tissue

Pincus You must have a radiation trapping enzyme then!

Dorfman Were your patients individuals who had recently had oestrogen therapy and had escaped from it?

Luft Your question is difficult to answer Dr Dorfman since most of our patients before hypophysectomy had been subjected to different kinds of treatment at the same time, for example irradiation and hormone administration

Crooke I would like to suggest to Dr Huseby that the dosage of oestrogen usually used clinically is an inhibitory dose You have to get down to something like 0 5 mg of stilboestrol daily or less to stimulate the pituitary function We have been doing that clinically too 1 mg a day is definitely inhibitory whereas 0 5 mg may be quite significantly stimulating to ICSH production

Huseby I am sure that is right as far as the gonadotrophins are concerned I believe that early in the treatment of human breast cancer Nathanson observed occasional regressions with doses in the area of 0 5 to 1 0 mg of stilboestrol per day

Crooke The line between those two doses is quite distinct

Huseby The doses Nathanson used may always have been over on the suppressing side I just do not know

Luft I think it might be of value in this connexion to study the effect of different dosages of stilboestrol on the male breast for instance in patients being treated for prostatic cancer We have done this in just a few ambulatory patients but it is too early even to predict the results

Gardner Mammary gland growth is inhibited in both the monkey and in the mouse by large doses of oestrogen One obtains a very small but nicely differentiated gland—a much smaller gland than in an animal that is getting a much smaller dose of oestrogen We do not know whether this takes place specifically at the pituitary level or at the mammary gland level, and we have never been able to design any experiments to find out The mammotrophic and the growth hormone will not correct the inhibiting effects of large doses of oestrogen

Dorfman Even if you use Dr Furth s preparation?

Gardner We have never used this preparation but a number of others both highly purified and not

Furth In an animal bearing grafted mammotrophic tumours the effect of oestrogen can be studied on both the mammotropes and the mammary gland Between the threshold dose of 10 μg of stilboestrol (given as a single pellet) and 1 mg there is a direct relation between mass of mammotrope and quantity of hormone administered Experiments are now being designed to use larger doses which in earlier studies proved to be toxic and to use natural hormone

Tata In experiments with induced thyroid tumours the pituitary



FIG 1 *Adrenogenital syndrome*—hyperplastic adrenal gland (weight 20 7 g) showing a uniform cortex composed of *compact* cells Zona reticularis and zona fasciculata appear as a single zone extending upwards to the zona glomerulosa which appears very prominent (Haematoxylin and eosin × 7)

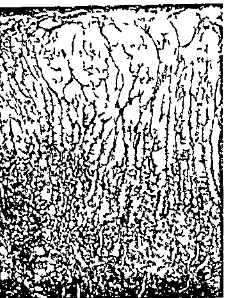


FIG 2 *Cushing's syndrome*—hyperplastic adrenal (weight 10 5 g) A broad zona reticularis is shown below and is composed of *compact* cells Above a prominent zona fasciculata consisting of *clear* cells is seen The zona glomerulosa lying under the capsule is not prominent (Haematoxylin and eosin × 15)

[Facing page 107

HYPERPLASIA AND TUMOURS OF THE HUMAN ADRENAL CORTEX HISTOLOGY ENZYMIC CHANGES AND CORTICOID PRODUCTION

T SYMINGTON * A R CURRIE * V J O DONNELL,† J K GRANT,‡ E G OASTLER§ and W G WHYTE§

IT is well established that Cushing s syndrome and the adrenogenital syndrome can result from carcinoma adenoma or hyperplasia of the adrenal cortex and the literature con tains many reports of Cushing s syndrome with adrenals apparently normal in size and morphology

The histological appearance of the hyperplastic gland in the two syndromes is accepted as showing considerable differences (Figs 1 and 2) Hyperplasia of the cortex in the adrenogenital syndrome shows a uniform picture in which the cells resemble those of the zona reticularis (Fig 1) It is not surprising that such an appearance was used to support the view that the zona reticularis of the adrenal gland was the site of androgen production Similarly the recognized char acteristic feature of the hyperplastic adrenal gland in Cush ing s syndrome is the large cell of the zona fasciculata (Fig 2) This appearance in Cushing s syndrome would seem to add weight to the concept that the zona fasciculata is the site of formation and of storage of the corticoid hormones

The view that the zona reticularis and zona fasciculata form androgens and corticoids respectively was apparently supported by the Vines fuchsinophil stain for androgens and the so called staining methods for ketosteroids in the zona fasciculata (Bennett 1940 Seligman and Ashbel 1952

*University Department of Pathology Glasgow Royal Infirmary
†University Department of Steroid Biochemistry Glasgow Royal Infirmary
‡D partment of Biochemistry University of Edinburgh
§Endocrine Unit Glasgow Royal Infirmary



Fig 1 *Adrenogenital syndrome*—hyperplastic adrenal gland (weight 20 7 g) showing a uniform cortex composed of *compact* cells Zona reticularis and zona fasciculata appear as a single zone extending upwards to the zona glomerulosa which appears very prominent (Hæmatoxylin and eosin × 75)

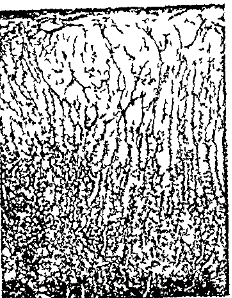


Fig 2 *Cushing's syndrome*—hyperplastic adrenal (weight 10 5 g) A broad zona reticularis is shown below and is composed of *compact* cells Above a prominent zona fasciculata consisting of *clear* cells is seen The zona glomerulosa lying under the capsule is not prominent (Hæmatoxylin and eosin × 45)

Facing page 10

Pearse 1953) However the non specificity and lack of re liability of both methods is now established Vines fuchsino phil stain is not seen consistently even in hyperplasia associ ated with the adrenogenital syndrome while the observations by Sayers (1950) Wolman and Greco (1952) and Karnovsky and Deane (1954) have provided proof that the ketosteroid stains demonstrate no more than the presence of unsaturated lipids In fact it is now clear that the steroid hormone content of the adrenal gland (Rogers and Williams 1947) is not demonstrated by any histochemical method

Biochemical studies using [^{14}C]acetate and cholesterol with whole adrenal gland slices homogenates and perfusion of the adrenal (Haines 1952 Hechter 1955) as well as urinary in vestigations (Bongiovanni 1953) have thrown light on the pathway of corticoid and androgen synthesis from acetate and cholesterol while histological studies (Yoffey 1952) have re newed interest in the zona reticularis as a functioning zone Far from being a senescent zone the reticularis appears to be the site of much activity In fact our observations suggest that the zona reticularis is a site of formation of corticoids and androgens from precursors such as acetate and cholesterol In conditions of stress the lipid precursor in the zona fasciculata (possibly cholesterol) is available and is used for steroid hormone synthesis

The data which prompt this conclusion are summarized and an attempt made to apply the results of some of our findings to the understanding of the role of the adrenals in Cushing s syndrome and in the adrenogenital syndrome

Effect of exogenous ACTH on morphology histochemistry and enzyme content of human adrenal cortex in cases of bilateral adrenalectomy for mammary carcinoma

Table I shows the plan of investigation The first gland was removed without any supportive hormone therapy and served as a control The patient was then left for a period of three weeks to allow the second gland to return to normal and

Table I

PLAN OF INVESTIGATIONS CARRIED OUT ON HUMAN ADRENAL GLANDS REMOVED BY TWO STAGE ADRENALECTOMY FOR BREAST CARCINOMA

1st Stage Adrenalectomy (Control)	*Interval about 3 weeks* →	*2nd Stage Adrenalectomy*
Day 1, 2, 3, 4—operation: No ACTH		Day 1, 2, 3, 4—operation: ACTH intramuscularly 100 units each day
Adrenal vein exposed if possible cannulated and blood collected		Blood collected as before
Adrenal gland removed part fixed for histology remainder ground for enzyme study		Adrenal gland removed part fixed for histology remainder ground for enzyme study

ACTH was then administered as shown The last injection of ACTH was given two hours before operation to ensure maximal stimulation of the gland The examinations carried out before and after ACTH stimulation are also given (Table II) and a summary of the changes found is shown in Table III

In the first or control gland large birefringent lipid globules which give the Schultz reaction for cholesterol are abundant in the zona fasciculata while in the zona reticularis the lipid

Table II

ADRENAL EXAMINATIONS BEFORE AND AFTER ACTH ADMINISTRATION

1	MORPHOLOGY →	Haematoxylin and Eosin
2	HISTOCHEMICAL CHANGES →	Acid and Alkaline Phosphatase Dehydrogenase—Symington Duguid and Davidson (1956) Ribonucleic Acid—Symington and Davidson (1956)
3	11β HYDROXYLATION →	Cortexone to Compound B (Adrenal homogenates)—Grant Symington and Duguid (1957)
4	ADRENAL VEIN EFFLUENT →	Blood flow amount and ratio Compound F/B—Grant Forrest and Symington (1957)

Table III

SUMMARY OF EFFECTS OF ACTH ON CELLS OF HUMAN ADRENAL CORTEX

	Before ACTH	*After ACTH*
Morphology	Clear	Compact
Lipid	Abundant	Scanty
Histochemistry		
Acid Phosphatase	Weak	Strong
Alkaline Phosphatase	Weak	Strong
Dehydrogenase (Krebs cycle)	Weak	Strong
Ribonucleic Acid	Weak	Strong
11β hydroxylation	<40 μg Cortico sterone/mg N/hr	60–180 μg
Ratio Compound F/Compound B in Adrenal Effluent	1 3 to 2 3	3 0 to 9 8

globules are small birefringence is weak and the Schultz re action for cholesterol is often absent There is a sharp histo logical division into zona reticularis and zona fasciculata Enzymes which can be demonstrated histochemically (acid and alkaline phosphatase and dehydrogenase of the Krebs cycle) are localized to the zona reticularis Likewise, ribo nucleic acid (RNA) is located in this zone and cannot be demonstrated histochemically in the zona fasciculata The cells of the fascicular zone are clear in appearance rich in lipid but poor in enzymes

The rate of blood flow in the adrenal vein in five patients in whom the vein was canalized varied from 0 39 to 1 9 ml / min and the ratio of Compound F/Compound B was 1 3 to 2 3 (Grant Forrest and Symington 1957) 11β Hydroxylation varied from patient to patient (Fig 3) In seven cases when the gland was considered histologically normal (0) (for histo logical grading see Currie and Symington 1955 Grant Symington and Duguid 1957) 11β hydroxylation varied from 23 to 44 μg corticosterone/mg N/hour with an average of 35 μg corticosterone The adrenal showed a + assessment in 5 cases and here the 11β hydroxylation results varied from 35 μg to 90 μg , in two cases when the adrenal picture was assessed at ++ 11β hydroxylation varied from 85 to 110 μg corticosterone/mg N/hour It is considered that in the seven

patients with adrenal assessments of + or ++, the high hydroxylation results are due to the liberation of increased amounts of endogenous ACTH

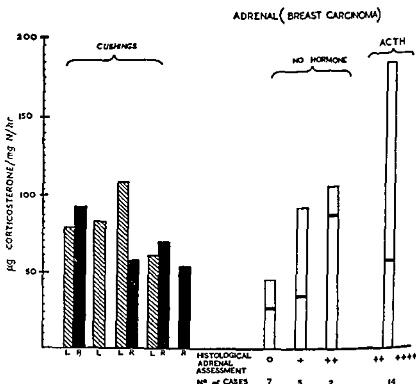


FIG 8 Comparison of the 11β hydroxylation of adrenal glands in Cushing's syndrome and of adrenal glands from patients with breast carcinoma In the latter the effect of ACTH administration is shown

When 400 mg ACTH was administered to the patient before removal of the second gland the most significant morphological and histochemical changes were seen in the cells of the zona fasciculata (Table III) They become *compact in* type, their cytoplasmic lipid small in amount and in some cases completely absent The cytoplasm of the cells is eosinophilic, rich in acid and alkaline phosphatase, dehydrogenase

and ribonucleic acid (Symington and Davidson 1956) There is a marked increase in the adrenal venous blood flow (2 0 to 6 0 ml /min) an increased output of Compound F and a rise in the ratio of Compound F/Compound B (3 0 to 9 8) Enzyme studies involving 11β hydroxylation were carried out on 14 cases with and without ACTH and a significant rise in the activity of the enzyme was seen in all cases This activity varied from 60 to 180 μg corticosterone/mg N/hour (Fig 3)

When the changes in the adrenal cortex before and after ACTH are summarized it is apparent that in the normal gland the enzymes stained by histochemical methods and RNA are concentrated in the cells of the zona reticularis while the zona fasciculata is rich in lipids but poor in enzymes and RNA When the gland is stimulated by ACTH the cells of the zona fasciculata nearest to the zona reticularis alter in type become compact and rich in enzymes and RNA If 200 to 300 mg ACTH are administered to the patient only the inner aspect of the zona fasciculata shows this alteration and by increasing the dosage the change can be effected in the whole cortex out to the zona glomerulosa which becomes compressed

Since alteration in the morphology and histochemistry of the cells of the zona fasciculata is associated with an increased output of cortisol and an increase in 11β hydroxylation it is very suggestive that the above changes are occurring in the cells of the zona fasciculata under the stimulus of ACTH Final proof however will require fractionation of the adrenal cortex into zona fasciculata and zona reticularis and demonstration of difference in their ability to effect the 11β hydroxylation of cortexone Nevertheless our observations suggest that endogenous ACTH acts on the cells of the zona reticularis In conditions of stress or after administration of ACTH when an elevated level of corticoids is required this is effected through the cells of the zona fasciculata commencing with those in the inner aspect of the zone and extending outwards gradually depending on the amount of ACTH administered

or on the degree of stress This results in an apparent widening of the zona reticularis The observation likewise indicates that functionally the zona reticularis and zona fasciculata should be considered as one rather than as two distinct zones

Application of above findings to lesions of the adrenal cortex causing adrenogenital and Cushing's syndromes

Adrenogenital syndrome The adrenogenital syndrome may result from adenoma, carcinoma or hyperplasia of the adrenal cortex The three cases we have studied were associated with adrenocortical hyperplasia The histological appearance of the gland is characteristic the cells are mainly compact in type usually extend up to the zona glomerulosa (Fig 1) although sometimes a narrow rim of clear cells (zona fasciculata) is seen lying below the glomerulosa (Fig 4) The appearance is similar to that seen in an ACTH treated or a stressed gland and is consistent with the finding of an increased concentration of ACTH in the blood (Sydnor *et al* 1953) In the adrenogenital syndrome there is a metabolic block at some point between 17 hydroxyprogesterone and Compound F as evidenced by the low amount of Compound F and its C_{21} metabolites in the blood and urine While Dorfman (1955) indicates that there is a relative defect in the 21 hydroxylating enzyme, Bongiovanni and Eberlein (1956) have provided evidence for a defect in $C_{(11)}$ hydroxylation in this syndrome It would appear that a study of the content of 11 and 21 hydroxylating enzymes of the adrenals is warranted

Cushing's syndrome likewise may result from adenoma carcinoma or hyperplasia of the adrenal cortex while a gland of normal size may be found Table IV shows the adrenal lesions observed in three reports A normal or hyperplastic gland was by far the most prevalent type in two series while in the third (Soffer *et al* 1955) it occurred in almost half the cases

When the 'normal" or hyperplastic glands of the Mayo Clinic (Sprague *et al* 1955) and the present series removed at



Fig 4 *Adreno genital syndrome*—hyperplastic adrenal (weight 17 g) A thin zone of clear cells (zona fasciculata) lies between a greatly enlarged zona reticularis and a very prominent zona glomerulosa (Haematoxylin and eosin × 75)



Fig *Cushing's syndrome*—hyperplastic adrenal gland (weight 16 g) found *post mortem* Note the columns of compact cells which extend up to the zona glomerulosa shown above Prominent cytolytic changes with complete disappearance of cords of cells are shown (Haematoxylin and eosin × 75)

Table IV

ADRENALS—CUSHING'S SYNDROME

Series	*No*	*Normal* (< 8 g)	*Hyperplasia*	*Adenoma*	*Carcinoma*
Soffer *et al* (1955)	33	10	5	8	10
Mayo Clinic (Sprague *et al* 1955)	88	34	35	14	5
Present series	20	7	8*	2	3
Total	141	51 (36%)	48 (34%)	24 (17%)	18 (13%)

Note the high percentage of cases (70%) in which the adrenal gland is normal or hyperplastic

*4 adrenals (post mortem)—all > 16 g
4 adrenals (operation)—all < 10.5 g

operation are reviewed (Table V) approximately half of them are within the limits of normality (each gland less than 8 g) while 90 to 100 per cent are less than 12 g. No operation gland in our series weighed more than 12 g. However when the five

Table V

NORMAL AND HYPERPLASTIC ADRENALS
CUSHING'S SYNDROME (OPERATION)
Adrenal Weights

Series	*No*	< 8 *g*	< 10 *g*	< 12 *g*	> 12 *g*
Mayo Clinic (Sprague *et al* 1955)	69	34 (50%)	54 (78%)	62 (90%)	7 (10%)
Present series	11	5 (45%)	9 (81%)	11 (100%)	—

non tumour adrenals observed *post mortem* are considered only one of them was normal in weight, the remainder were hyperplastic and each gland weighed between 16 and 32 g

A distinct difference occurs in the histological appearance of glands removed at operation and *post mortem* The adrenal from operation shows a prominent zona reticularis which extends outwards to replace the cells of the zona fasciculata in varying degree (Fig 2) The cells of the zona reticularis are compact in type, depleted of lipid but rich in RNA and in acid and alkaline phosphatase The zona fasciculata cells are clear, larger than normal and poor in enzymes This appearance occurs in the adrenal removed at operation whether the gland is "normal" in size or hyperplastic and from what has been described in the response to ACTH it is apparent that in both instances the significant feature is the increased width of the zona reticularis When ACTH is administered to a patient with Cushing s syndrome and a "normal" or hyperplastic gland is removed subsequently at operation it is found that the adrenal has responded in the same manner as in a normal subject There is conversion of the clear cells of zona fasciculata to the compact type of cell and the whole cortex presents a uniform appearance This is also the picture found in the hyperplastic adrenal of Cushing s syndrome found *post mortem* (Fig 5) Compact cells of variable size extend outwards to the zona glomerulosa which becomes compressed against the capsule Degenerative changes such as cytolysis and lumen formation are frequently seen and resemble to a lesser degree the changes found in some cases in conditions of stress (Symington *et al* 1955)

11β Hydroxylation studies were carried out on homogenized adrenal glands of normal weight from five patients subjected to adrenalectomy Both right and left glands were examined in three and a left and a right gland only in two patients (Fig 3) 11β Hydroxylation is estimated as μg corticosterone/mg N/hour formed from cortexone This varied from 60 to 105 and was well above the upper limit of normality (44) observed when the adrenal gland was considered normal histologically

(0 assessment) Since 11β hydroxylation results between 53 and 110 (Fig 3) were observed in the adrenals (+ to ++ assessment) of seven breast cancer patients who had had no hormone therapy before operation the finding of similar hydroxylation results in cases of Cushing s syndrome becomes less significant especially as canalization of the adrenal vein was not carried out in any of the cases Nevertheless, it may be concluded that high 11β hydroxylation results (60–105) in a homogenized adrenal from a patient who has received no ACTH treatment are not necessarily indicative of a Cushing s syndrome but if a patient exhibiting the clinical signs of Cushing s syndrome at operation has an adrenal gland of *normal size it will show a large zona reticularis and a high* 11β hydroxylation capacity The test is therefore one that should be carried out since it helps to explain the large number of cases of Cushing s syndrome recorded with adrenals of normal size (Crooke 1953 Kuppermann *et al* 1953 Soffer *et al* 1955) Such observations once more focus attention on the expressions of Albright that in cases where the adrenal cortices have been found normal the author questions either the diagnosis or the interpretation of adrenal histology Again in his Harvey Lectures (1942–3) Albright states if the adrenal is normal in size the patient has hyperfunction which routine gross or microscopic examination of the gland does not reveal

Remissions in Cushing s syndrome

If as has been shown the zona reticularis is the active zone of steroid hormone production any lesion causing replacement of or interference with the function of this zone in Cushing s syndrome might be expected to cause remissions of the disease Some degree of lipid replacement of the zona reticularis is seen in most cases of the disease but in two patients showing remissions extensive replacement of the cells in this zone was observed While remissions have been noted following pituitary irradiation (Soffer *et al* 1955 Cappell 1957) or testosterone therapy the state of the

non tumour adrenals observed *post mortem* are considered only one of them was normal in weight the remainder were hyperplastic and each gland weighed between 16 and 32 g

A distinct difference occurs in the histological appearance of glands removed at operation and *post mortem* The adrenal from operation shows a prominent zona reticularis which extends outwards to replace the cells of the zona fasciculata in varying degree (Fig 2) The cells of the zona reticularis are compact in type depleted of lipid but rich in RNA and in acid and alkaline phosphatase The zona fasciculata cells are clear larger than normal and poor in enzymes This appearance occurs in the adrenal removed at operation whether the gland is normal ' in size or hyperplastic and, from what has been described in the response to ACTH it is apparent that in both instances the significant feature is the increased width of the zona reticularis When ACTH is administered to a patient with Cushing s syndrome and a normal or hyperplastic gland is removed subsequently at operation it is found that the adrenal has responded in the same manner as in a normal subject There is conversion of the clear cells of zona fasciculata to the compact type of cell and the whole cortex presents a uniform appearance This is also the picture found in the hyperplastic adrenal of Cushing s syndrome found *post mortem* (Fig 5) Compact cells of variable size extend outwards to the zona glomerulosa which becomes compressed against the capsule Degenerative changes such as cytolysis and lumen formation are frequently seen and resemble to a lesser degree the changes found in some cases in conditions of stress (Symington *et al* 1955)

11β Hydroxylation studies were carried out on homogenized adrenal glands of normal weight from five patients subjected to adrenalectomy Both right and left glands were examined in three and a left and a right gland only in two patients (Fig 3) 11β Hydroxylation is estimated as μg corticosterone/mg N/hour formed from cortexone This varied from 60 to 105 and was well above the upper limit of normality (44) observed when the adrenal gland was considered normal histologically



Fig 6 *Cushing's syndrome* — adrenal adenoma (weight 2 0 g) The cells are uniform in size and have an alveolar arrangement (Haematoxylin and eosin × 112 .)



Fig 7 *Cushing's syndrome* Bizarre nuclear forms from same specimen as Fig 6 The nuclei have a vesicular appearance Compare with Fig 10 (Haematoxylin and eosin × 187 .)

adrenals under such conditions is unknown Nevertheless the possibility exists that the lesion may be one of lipid replacement, as described here

Cortical tumours causing Cushing's syndrome

Table IV shows the incidence of cortical tumours found in three recorded series In Soffer s group carcinoma was slightly commoner while adenoma occurred more frequently in the Mayo Clinic series Five cases occurred in our series, two were adenomas and three carcinomas *Adenomas* vary greatly in size from 5 to approximately 200 g In the Mayo Clinic group the range which includes carcinomas was much narrower 5–40 g The tumour is usually well encapsulated, brown or white in colour with occasional yellow flecks of adrenocortical lipids scattered throughout the growth Areas of necrosis are present in the large tumours but in the smaller ones necrosis is minimal The histological appearance varies considerably and most of the cell types described in the normal or hyperplastic glands are seen The growth may be composed exclusively of compact cells, of uniform size, arranged in columns similar to those seen in the zona fasciculata of a stressed or ACTH treated adrenal gland or the cells may have a more alveolar arrangement (Fig 6) The nuclei are generally larger than normal and uniform in size but frequently nuclear pleomorphism is prominent and bizarre nuclear forms are seen (Fig 7) Such large nuclei have a vesicular appearance and show no great disproportion in nuclear cytoplasmic ratio Scattered throughout some tumours are groups of large cells *either compact in appearance or clear in type and full of lipid* They resemble the cells seen in the hyperplastic gland (Fig 8) It is not uncommon to see small clear cells similar to those present in the normal zona fasciculata Mitotic figures are uncommon

In contrast to adrenal adenoma *carcinoma* causing Cushing s syndrome is much larger is usually globular in shape and surrounded by a capsule which may or may not be adherent to an adjacent organ It may be soft in consistency or friable



FIG 10 *Cushing's syndrome* From the same gland as Fig 9 showing large giant cells with a hyperchromatic nucleus (Haematoxylin and eosin × 187 a)



Fig. 8 *Cushing's syndrome* Another section of the tumour showing large foamy cells (Haematoxylin and eosin × 187.5)



Fig. 9 *Cushing's syndrome* Adrenal adenoma—showing the abrupt transition in cellular pattern. Small cells with hyperchromatic nuclei and mitotic figures are present (above). Larger cells with more vesicular nuclei and less mitosis are seen below. (Haematoxylin and eosin × 11[illegible])

and necrotic and divided into lobules by prolongations of the capsule into it Microscopically the tumour consists of anastomosing columns of compact cells with granular acidophilic cytoplasm and ovoid vesicular nuclei Many nuclei are hyperchromatic and mitotic figures are numerous Such areas may merge with others composed of large clear cells (Fig 9) A not infrequent finding is the presence of giant cells of strikingly large size (Fig 10) Their cytoplasm is granular and acidophilic and they have a centrally placed hyperchromatic nucleus The growth is usually supplied by abundant capillary blood vessels

Few histochemical studies have been carried out on adrenal tumours associated with Cushing s syndrome (Seligman and Ashbel 1952) and since they are uncommon little opportunity exists for 11β hydroxylation studies Nevertheless such enzyme studies are essential for the elucidation of many of the problems of adrenal tumour pathology

Summary

It is apparent that the zona reticularis and zona fasciculata should not be considered as two separate functional zones but rather that the zona reticularis is an active zone in the biosynthesis of corticoids and androgens possibly from acetate and/or cholesterol The zona fasciculata is a storage zone for steroid precursors such as cholesterol When steroid hormone synthesis is increased (after ACTH or in conditions of stress) cholesterol is utilized and the cells of the zona fasciculata undergo the morphological and enzymic changes described That steroid hormones are produced under these conditions has been shown by canalization of the adrenal vein and estimation of the amount and ratio of Compounds F and B (Grant Forrest and Symington 1957) and androgens (Pincus and Romanoff 1955) The possibility that the same adrenal cell can produce corticoids and androgens is not unreasonable if we consider the pathway of steroid synthesis as suggested by Dorfman (1955) This concept is strengthened from a study

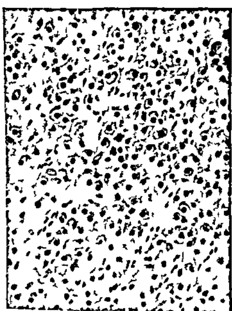


FIG 11a



FIG 11b

FIG 11a and 11b The compact cell of the hyperplastic adrenal gland in the adreno-genital syndrome (Fig 11a) and Cushing's syndrome (Fig 11b) is shown In one instance (Fig 11a) androgens are predominantly formed in the other (Fig 11b) corticoids or corticoids and androgens (Hæmatoxylin and eosin × 187 5)

BONGIOVANNI A. M and EBERLEIN W R (1956) *In* Adrenal Function in Infants and Children A Symposium ed L I Gardner p 76 New York Grune & Stratton
CAPPELL, D F (1957) Personal communication
CROOKE A C (1953) *Post Grad med J* 29 179
CURRIE A R and SYMINGTON T (1955) Ciba Foundation Colloquia on Endocrinology 8 396 London J & A Churchill Ltd
DORFMAN R I (1955) Ciba Foundation Colloquia on Endocrinology 8 112 London J & A Churchill Ltd
GRANT J K FORREST A M P and SYMINGTON T (1957) *Acta endocr Copenhagen* 26 195
GRANT J K SYMINGTON T and DUGUID W P (1957) *J clin Endocrin Metab* 17 933
HAINES W J (1952) *Recent Progr Hormone Res* 7 255
HECHTER O (1955) Ciba Foundation Colloquia on Endocrinology 8 161 London J & A Churchill Ltd
KARNOVSKY M L and DEANE H W (1954) *J Histochem Cytochem* 2 478
KUPPERMANN H S BERNSTEIN A FORBES A P COPE O and ALBRIGHT F (1953) *J clin Endocrin Metab* 13 154
PEARSE A G E (1953) *In* Histochemistry p 212 London J & A Churchill Ltd
PINCUS G and ROMANOFF E B (1955) Ciba Foundation Colloquia on Endocrinology 8 97 London J & A Churchill Ltd
ROGERS W F Jr and WILLIAMS R H (1947) *Arch Path (Lab Med)* 44 126
SAYERS G (1950) *Physiol Rev* 30 241
SELIGMAN A M and ASHBEL, R (1952) *Endocrinology* 50 338
SOFFER L J EISENBERG J IANNACCONE A and GABRILOVE J L (1955) Ciba Foundation Colloquia on Endocrinology 8 487 London J & A Churchill Ltd
SPRAGUE R G RANDALL, R V SALASSA, R M SCHOLTZ D A PRIESTLY J T WALTERS W and BULBULIAN A H (1955) A.M.A Scientific Exhibits p 315 New York Grune & Stratton
SYDNOR K L KELLY V C RAILE R B ELY R S and SAYERS G (1953) *Proc Soc exp Biol N Y* 82 596
SYMINGTON T CURRIE, A R CURRAN R C and DAVIDSON J N (1955) Ciba Foundation Colloquia on Endocrinology 8 70 London J & A Churchill Ltd
SYMINGTON T and DAVIDSON J N (1956) *Scot med J* 1 15
SYMINGTON T DUGUID W P and DAVIDSON J N (1956) *J clin Endocrin Metab* 16 580
WOLMAN M and GRECO J (1952) *Stain Tech* 27 317
YOFFEY J M (1952) *In* The Suprarenal Cortex p 131 London Butterworths Scientific Publications

of the 'normal" and hyperplastic glands in Cushing s syndrome and the hyperplasia of the adrenal in the adrenogenital syndrome In the two syndromes the compact cell having the morphological and histochemical characters described above (Fig 11, *a* and *b*) *can produce corticoids and androgens and* in a case of Cushing's ('mixed') with androgenic effects both types of steroid are produced

Attention has been drawn to the 11β hydroxylation of cortexone and the value of this transformation in the assessment of activity in the 'normal' adrenal associated with Cushing's syndrome Although equally high levels can be found in adrenals removed from patients with breast cancer who have received no supportive hormone therapy such glands are known to be active from the high corticoid content of adrenal venous blood While our observations thus confirm the views *of Albright that the normal size adrenal of Cushing s syn*drome appears to be a hyperactive gland it may be due simply to an abnormal sensitivity of the gland to endogenous ACTH

Although varied forms of the compact cell are seen in adenoma and carcinoma causing Cushing s syndrome no histochemical or steroid hydroxylation observations have been made on them However only operation material is of value for hydroxylation studies since previous experience has shown that post mortem glands even those collected within two hours of death are unsatisfactory (Grant, Symington and Duguid 1957)

In distinguishing between adenoma and carcinoma at present only morphological studies are available and in carcinoma the abundance of mitosis and the hyperchromatic appearance of the giant cells with nuclear and cytoplasm dis*proportion* the vascularity of the tumour and to some extent the capsular infiltration are aspects of the growth which point to malignancy

REFERENCES

ALBRIGHT F (1942–3) *Harvey Lect* 38 123
BENNETT H S (1940) *Amer J Anat* 67 151
BONGIOVANNI A M (1953) *Johns Hopk Hosp Bull* 92 36

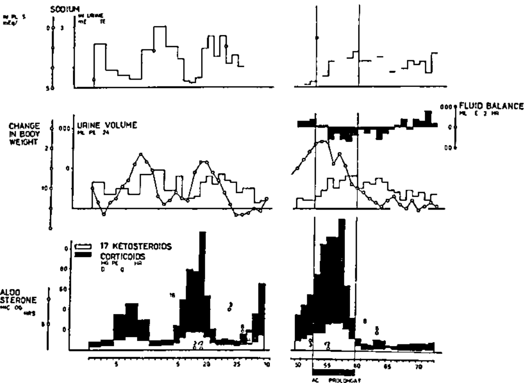


Fig 1 (Luft) Spontaneous and ACTH induced changes in plasma and urinary sodium urine volume and body weight correlated with the urinary excretion of aldosterone corticoids and 17 ketosteroids in a 5[illegible] year old woman with hyperadrenocorticism

DISCUSSION

Luft In connexion with Prof Symington s paper I would like to present briefly some data of ours (Hokfelt Luft Nilsson and Sekkenes) on a case of hyperadrenocorticism associated with marked oedema appearing periodically with intervals of 8–30 days The patient is a 55 year old housewife with symptoms of her present illness during the last four to five years The characteristic features of her disease are (Fig 1)

during 3–4 days weight gain of 5–10 pounds in connexion with appearance of generalized oedema
at the peak of weight gain blurring of vision and mental depression
the period of weight gain is followed by a period of 3–4 days of weight loss connected with disappearance of oedema
the weight gain is accompanied by retention of water sodium and chloride and a loss of potassium an increase in plasma sodium and chloride and a decrease in plasma potassium a decrease in haematocrit and circulating eosinophils
the weight loss is accompanied by the opposite changes
during the weight gain the blood pressure reaches values of 195/145 mm Hg while it is normal during the stable days

During the period of weight gain there is a simultaneous marked increase in total 17 ketogenic corticoids (see Appleby *et al* *(1955) Biochem J* 60 453) up to values of 116 mg per 24 hours and some increase in neutral 17 ketosteroids The urinary excretion of aldosterone (see Neher and Wettstein (1956) *J clin Invest* 35 800) is not measurable at the peak of water retention and reaches somewhat increased values when the body weight is at its lowest

A semiquantitative estimation of corticoid compounds in urine when the corticoids were at their highest and at their lowest as well as during administration of ACTH gave the results presented in Table I During

Table I (Luft)

IDENTIFIED (MG /24 HR) AND INDICATED (X) URINARY α KETOLIC METABOLITES SEMIQUANTITATIVE ESTIMATION

Total corticoids	H_4F	H_4E	*F*	*E*	H_4S	H_4B	*S*	*B*
6	0 03	0 03	X	X	–	–	–	?
75	1 5	0 8	1 5	0 4	0 2	X	?	X
112 (ACTH 40 i u /24 hr)	2 5	1 0	4 4	1 0	X	?	?	X

Luft As far as I know Prof Groen our patient was not taking any of the drugs or sweets that might induce water and sodium retention She was kept in our metabolic ward and was not allowed to receive food or drugs from outside I want to emphasize that our patient had a markedly enlarged sella turcica on X ray The sella was of a size that could only be explained by the presence of a pituitary tumour

Crooke I was not suggesting that her pituitary was normal I suggested that the rapid fluctuations in sodium and water which you describe are much more common than is generally recognized

Groen Did you test the urine for liquorice or Pyramidone?

Luft No

Dorfman Dr Crooke what were the corticoid values in the migraine patients?

Crooke Dr Hay asked if we would co operate with this but we have not had time to get any results yet

Dorfman These are enormous values that Dr Luft reports and one of the most remarkable things about the paper chromatography analysis is this enormous increase in cortisol and cortisone in the face of a minimum increase in the tetrahydrocortisone and tetrahydro cortisol

Gray The other feature was that even after ACTH the reduced products did not increase either I would have thought that this point together with the one you made Dr Dorfman ruled out any question of an interfering compound

Dorfman There is also the question of this type of extreme weakness or Addisonian crisis which this patient had In other words there is a relative deficiency of cortisol cortisone and those products The analysis leads me to suspect too that there is a very high amount of 11 deoxy cortisol reflected by the tetrahydro derivative It seems that in the unstimulated state this person is failing to 11β hydroxylate the steroids and therefore the steroids which give a good Porter Silber reaction are not doing the patient very much good

Furth In general your approach is the same as ours Prof Symington We are both attempting to correlate morphology with function However I should like to challenge your conclusions There are about four functional systems in the adrenal the androgenic oestrogenic gluco corticoid and mineralocorticoid systems The American school relates the mineralocorticoids to the glomerulosa which is not under ACTH control so there is a clear cut cellular distinction Some consider the androgenic zone juxta medullary in location while it is accepted that the fasciculate zone is concerned with secretion of glucocorticoids Secretion of gonadal hormones in mice is attributed to clumps of cells in the subcortical region Two ways of regulation are conceivable it is either intracellular on the enzyme level or the different functions are performed by different cells With respect to sites and regulation of adrenal function I am utterly lost Glucocorticoids are believed to be regulated by ACTH mineralocorticoids by sodium/potassium levels Conceivably gonadal hormonal production of the pituitary is influenced by gonadotrophins You suppose that there is just one type of cell and regulation

the peak of corticoid excretion remarkably large quantities of free cortisol and cortisone are excreted Tetrahydro S is also found in considerable amounts while corticosterone is present in smaller amounts Rather large quantities of a steroid more polar than tetrahydrocortisol presumably Δ^4 *pregnene 11β 17α 20β 21 tetrol 3 one* is also present For identification the following tests were used ultraviolet light absorption at 245 mμ mobility in two or more solvent systems reaction with alkaline blue tetrazolium sodafluorescence phenylhydrazine reaction spectrometry in sulphuric acid

In this patient examination of the adrenal glands by perirenal insufflation of oxygen revealed glands of normal size and shape On the other hand X ray examination of the skull showed an enlarged sella turcica with decalcification The probable diagnosis is therefore a pituitary tumour In order to elucidate the case further two determinations of ACTH in blood have been performed according to the method of Sydnor and co workers (1953 *J clin Endocrin Metab* 13 891) In none of the blood samples could any measurable amounts of ACTH be detected Despite this we are of the opinion that the pathological substrate is an ACTH producing pituitary tumour The reason for the periodical appearance of the characteristic features remains obscure

Crooke Dr Hay in Birmingham has shown me a collection of data on many patients which remind me of yours Dr Luft All his patients have migraine and some have frequent and severe attacks They get sodium and water retention during the attacks exactly like you showed It is apparently very common people just have not looked for it I think that the fluctuations which you describe may be a related phenomenon

Groen Could the patient have taken some substance which would produce water retention with the sodium and chloride retention and potassium excretion? Several of these cases which we have seen lately in the Netherlands and in Belgium were discovered to be liquorice addicts Liquorice as you know is a favourite sweet in Holland and by taking sufficient amounts many normal individuals and patients produce this syndrome Either the diagnosis is made from the history or one can test the urine in a very simple way add some sulphuric acid and the colour turns red This red colour interferes with the determination of 17 hydroxycorticoids if you use the Porter Silber principle Not only does it increase the background colour but it also increases the 17 hydroxycorticoid values So one may get a pseudo increase in excretion of 17 hydroxycorticoids in a patient who has been taking liquorice and shows water and sodium retention Pyramidone (aminopyrine) or butazolidine will do more or less the same thing in some individuals even to a marked degree and these substances can also be detected quite easily in the urine So if your patient is mentally disturbed or suffers from migraine just check whether she has taken some drug before making a diagnosis of a new syndrome I remember one patient who had negative reactions in the urine with this syndrome of periodic water and sodium retention but she was mentally so disturbed that I never trusted that she had not taken some drug unknown to me

Finkelstein We encountered several cases of Cushing s syndrome with apparently normal adrenals Their hormonal urine analysis for keto steroids corticosteroids etc did not show anything unusual In a few of these cases however we found the compound I mentioned yesterday pregnane 3α 17α 20α triol 11 one and Dr Gallagher recently isolated it from a classical case of Cushing s Since as I have emphasized before pregnanetriolone is not excreted by normal individuals a defect in $C_{(21)}$ hydroxylation must be involved in those cases of Cushing s with the so called adrenal hyperplasia Since on visual inspection the adrenals appear normal in many of these cases and on the other hand unusual steroid compounds such as pregnanetriolone are being excreted I would stop calling these cases adrenal hyperplasia and term them instead adrenal heterofunction What we are actually seeing is a different way of biosynthesis and not a mere increase in size and activity

Preedy How much blood are the Scottish surgeons able to obtain from the adrenal vein? It should be possible if the oestrogen concen tration is at all reasonable to measure the oestrogens in adrenal vein blood If you can get sufficient blood to obtain 0 05 μg of each of the oestrogens you ought to be able to make the estimation fairly easily

O Donnell The surgeons can provide about 20 to 100 ml of blood from the adrenal vein depending on the circumstances and I agree with Dr Preedy that it would be reasonable to attempt to measure oestro gens in that blood

About the 17 and 21 hydroxylation in adrenal homogenates from breast cancer cases before and after ACTH Dr Grant has found no difference It was only 11β hydroxylation that was found to increase after treatment with ACTH However these results on the 17 and 21 hydroxylation are preliminary

is intracellular What you have actually shown is function and stimulation but not specificity of function What we need is identification of specific functions by histochemical localization of enzymes performing the key functions in steroidogenesis or identification of the hormones themselves

Symington I am glad you brought up that point I was very careful and did not mention anything about mineralocorticoids It is now well established from the work of Ayres and co workers (1956 *Biochem J* 63 19P) on the ox gland that the zona glomerulosa is the site of production of aldosterone in that animal and it now seems quite definite that the zona glomerulosa is a distinct zone We are limited to androgens corticoids and possibly oestrogens While we have direct and indirect evidence that the adrenal liberates oestrone this steroid has not been identified in adrenal effluent We are left with corticoids and androgens The so-called ketosteroid reactions of Bennett (1940) and Seligman and Ashbel (1952) are not specific and observations by Wolman and Greco (1952) Sayers (1950) and Karnovsky and Deane (1954) have *shown that the above ketosteroid methods are staining no more than I* am doing with Sudan IV I think we can forget about trying to visualize those hormones by histochemical methods the approach must surely be microchemical as against histochemical—by using the freeze cabinets and a tissue slicer Using these techniques we have tried to break up the adrenal into the two different zones (*zona reticularis* and *zona fasciculata*) If we could get sufficient material from these two zones we would be able to tell whether or not the 11 17 and 21 hydroxylating enzymes vary in the different zones This has been our problem

Pincus Have you tried any adrenogenital syndrome tissue to discover its steroidogenic capacity?

Symington No

Pincus It seems to me from what you showed that there is no obvious difference in these so called secretory cells

Symington Yes I think that is quite true One point Dr Pincus is that our chance of ever getting a hyperplasia from the adrenogenital syndrome is pretty poor due to the treatment with cortisol In fact it is rather surprising that we were able to show these pictures at all

Dorfman Regarding the biosynthesis of the hormones in the different zones Drs Péron and Koritz in our laboratory recently had the opportunity to work with rat adrenal glomerulosa as a result of adrenal enucleation To our surprise and interest this particular zone produced corticosterone and responded to ACTH as well as the whole gland

Luft Prof Symington would you consider the first adrenal you removed as normal taking into consideration that the patient had been submitted to anaesthesia and to quite severe surgical stress?

Symington I would say that a normal adrenal is a very very rare gland and that it is one with a small reticularis and a big fasciculata that is the nearest I can get to the so called normal I agree it is very rare and very difficult to pick it up at post mortem

Crooke But it is a very interesting control for the other kind and that is what you are primarily concerned with

An experimental test of the idea of adrenal ovary relation ship appears to have been first performed by Miss H E Feodossiew at the University of Kazan and published in 1906 The experiment seems to have eluded a number of workers in the United States It seems that Professor Lubinov and his student Miss Feodossiew clearly understood that a relation ship existed between the ovary and the adrenal cortex In exploring this they used dogs which were castrated and then autopsied at definite intervals of months The adrenal glands were studied and adrenocortical hyperplasia was des cribed The hyperplasia started in the region of the zona glomerulosa and progressed to the zona fasciculata The *hyperplasia in both layers was focal i e in islands* The growths reached and penetrated the medulla in one direction and progressed through the capsule in the other forming in the latter case a mushroom like growth which remained united to the cells of the peripheral layer Increase in the number of mitotic figures was noted All of this sounds remarkably modern and similar to our present concept of nodular hyper plasia of the adrenal cortex

In the older literature however reports of adrenocortical tumours in experimental animals are extremely rare Slye Holmes and Wells (1921) found four adrenocortical tumours in the autopsy of 33 000 experimental mice McCoy (1909) found no adrenal tumours in 100 000 wild rats Woolley and Wherry (1911) found no adrenal tumours in 23 000 wild rats Curtis Bullock and Dunning (1931) reported only one case of adrenal tumour in 31 868 ageing rats of many strains

A number of years ago we started experimentation to try to lower the incidence of breast cancer in strain DBA mice a relatively high incidence strain whether the animals were breeding or virgin animals This turned into an adrenocortical study in an interesting way (Woolley Fekete and Little 1939)

In order to reduce breast cancer incidence we tried to extend the work of Loeb (1919) Murray (1927 1928) and Cori (1926 1927) who had reduced breast cancer incidence by ovariectomy Since earlier and earlier ovariectomy had

TUMOURS OF THE ADRENAL CORTEX*

GEORGE W WOOLLEY

Division of Steroid Biology Sloan Kettering Institute for Cancer Research and the Sloan Kettering Division Cornell University Medical College New York

THE adrenal as is well known, consists of two ductless glands in close physical association In lower forms such as amphibia the two primary tissues are separated into distinct bodies In birds the cortical and medullary tissue, so called, is interlaced In higher forms there is a true cortical and medullary relationship

In a mammal such as the mouse the anlagen of the cortex and medulla are separate when first found at about 13 days of embryonic life As development proceeds the medullary elements penetrate the cortical body and shortly before birth take up a central or medullary position In early life a transitory zone the so called X zone develops between the cortex and medulla This area or zone is closely related to the cortex This region shows variations in width and persistence related to sex, age and strain At one time it was thought that this zone might be the site of origin of sex hormone producing adrenocortical tumours

There is evidence that adrenocortical tumours were recorded in necropsy findings a century and a half ago and earlier in man Ideas regarding the aetiological relationships of adrenocortical tumours seem to have been first recorded only 50 to 60 years ago In 1891 Marchand described a case in which a human female hermaphrodite had on autopsy atrophied ovaries and greatly enlarged adrenals In a similar case Creccio (1865) found the adrenals had increased to the size of the kidneys

* This investigation was supported in part by a research grant (C 1796) from the National Cancer Institute of the National Institutes of Health Public Health Service and in part by an Institutional Research Grant from the American Cancer Society Inc

An experimental test of the idea of adrenal ovary relationship appears to have been first performed by Miss H E Feodossiew at the University of Kazan and published in 1906 The experiment seems to have eluded a number of workers in the United States It seems that Professor Lubinov and his student Miss Feodossiew clearly understood that a relationship existed between the ovary and the adrenal cortex In exploring this they used dogs which were castrated and then autopsied at definite intervals of months The adrenal glands were studied and adrenocortical hyperplasia was described The hyperplasia started in the region of the zona glomerulosa and progressed to the zona fasciculata The hyperplasia in both layers was focal i e in islands The growths reached and penetrated the medulla in one direction and progressed through the capsule in the other forming in the latter case a mushroom like growth which remained united to the cells of the peripheral layer Increase in the number of mitotic figures was noted All of this sounds remarkably modern and similar to our present concept of nodular hyperplasia of the adrenal cortex

In the older literature however reports of adrenocortical tumours in experimental animals are extremely rare Slye Holmes and Wells (1921) found four adrenocortical tumours in the autopsy of 33 000 experimental mice McCoy (1909) found no adrenal tumours in 100 000 wild rats Woolley and Wherry (1911) found no adrenal tumours in 23 000 wild rats Curtis Bullock and Dunning (1931) reported only one case of adrenal tumour in 31 868 ageing rats of many strains

A number of years ago we started experimentation to try to lower the incidence of breast cancer in strain DBA mice a relatively high incidence strain whether the animals were breeding or virgin animals This turned into an adrenocortical study in an interesting way (Woolley Fekete and Little 1939)

In order to reduce breast cancer incidence we tried to extend the work of Loeb (1919) Murray (1927 1928) and Cori (1926 1927) who had reduced breast cancer incidence by ovariectomy Since earlier and earlier ovariectomy had

TUMOURS OF THE ADRENAL CORTEX*

GEORGE W WOOLLEY

Division of Steroid Biology Sloan Kettering Institute for Cancer Research and the Sloan Kettering Division Cornell University Medical College New York

THE adrenal, as is well known consists of two ductless glands in close physical association In lower forms such as amphibia the two primary tissues are separated into distinct bodies In birds the cortical and medullary tissue so called is interlaced In higher forms there is a true cortical and medullary relationship

In a mammal such as the mouse the anlagen of the cortex *and medulla are separate when first found at about 13 days* of embryonic life As development proceeds the medullary elements penetrate the cortical body and shortly before birth take up a central or medullary position In early life a transitory zone the so called X zone develops between the cortex and medulla This area or zone is closely related to the cortex This region shows variations in width and in per sistence related to sex age and strain At one time it was thought that this zone might be the site of origin of sex hormone producing adrenocortical tumours

There is evidence that adrenocortical tumours were recorded in necropsy findings a century and a half ago and earlier in man Ideas regarding the aetiological relationships of adrenocortical tumours seem to have been first recorded only *50 to 60 years ago In 1891 Marchand described a case* in which a human female hermaphrodite had on autopsy atrophied ovaries and greatly enlarged adrenals In a similar case Creccio (1865) found the adrenals had increased *to the* size of the kidneys

* This investigation was supported in part by a research grant (C 1796) from the National Cancer Institute of the National Institutes of Health Public Health Service and in part by an Institutional Research Grant from the American Cancer Society Inc

produce predominantly oestrogen or predominantly androgen or different proportions of the two was associated with the tumours in transplant generations

The effect of gonadectomy was subsequently studied in six reciprocal crosses between different strains of mice Some 1 700 offspring were produced and studied with autopsies *beginning at 15 days of age and continuing at monthly* intervals through 24 months of age or beyond These emphasized the high frequency of association between adrenocortical tumours and accessory reproductive organ changes typical of those associated with sex hormone production in experimental mice

Having observed oestrous cycles in DBA and CE mice after gonadectomy we became interested in whether or not these gonadectomized females might mate as a result of sex hormone secreted by their adrenal cortices Christy Dickie and Woolley (1950) observed that the gonadectomized mice would mate in a normal manner when in oestrus and that the female mating response to the male is normal in all respects It seemed that the adrenal neoplasms were efficient substitutes for the gonads in this regard

In one set of experiments (Dickie and Woolley 1949) male and female mice of the F_1 generation of reciprocal crosses of strains C57BL DBA CE A and C3H were gonadectomized neonatally and allowed to age without further treatment Virgin females and unmated males were used as control animals At autopsy 14 to 26 months of age in the experimental groups pituitary abnormalities occurred Adrenocortical nodular hyperplasias and carcinomas were also present Histological analysis of the hypophyses showed anterior lobe basophilia and basophilic adenomata The most striking change concurrent with the hypophysial tumours was extensive differentiation and over development of the mammary glands This change became a criterion for predicting the presence or absence of the hypophysial changes It was interpreted that in the combination of pituitary adrenal dysfunction substances were secreted similar to gonadotrophic

tended to reduce the incidence more and more we ovariectomized a group immediately after birth and were surprised to find that the animals so gonadectomized still had breast cancer when they had aged In addition the animals had vaginae with cornified epithelium enlarged uteri etc No ovaries were present but the adrenals were noted to be abnormal The adrenal abnormality was carefully worked out and described as nodular hyperplasia of the adrenal cortex There is evidence that sex like hormones of oestrogen type were produced by these modified tumorous adrenals

In general, the simplest and most common adrenocortical tumour type in mice is nodular hyperplasia The occurrence of adenomas has been remarked on as has that of carcinomas Accessory reproductive organ status indicates that endocrine secretion of a sex hormone nature may be associated with each of these tumour types in the mouse and in certain other animals Other adrenocortical hormones may also be present in excess in certain instances as is shown below

In our experiments only biological methods are employed to determine the quality and quantity of hormones produced by the adrenocortical and adrenocortical and pituitary tumours As well as the accessory reproductive organs uterus vagina, mammary glands preputial glands vesicular glands and prostates the submaxillary glands and kidneys were found to be useful sites for these evaluations

For several years we carried out systematic examination of the growth and endocrine changes in different inbred strains of mice following gonadectomy These experimental animals had varying changes toward tumour in their adrenal cortices The tumour type varied from strain to strain but was uniform within strains Some such as strain DBA produced only oestrogenic hormones when evaluated from accessory reproductive organ changes Others such as strain A produced androgenic hormones while a strain such as CE gave evidence of having both oestrogenic and androgenic hormones

Tumours of the various secretory types from strain CE were *transplanted into* suitable host animals and the ability to

3 methyl dehydrodoisynolic acid was non effective when absorption was at the rate of 0 014 mg /day

Dehydro*iso*androsterone, as absorbed from one pellet was not effective When absorption was continuous from three pellets (1 mg /day as compared to 0 4 mg /day) it exhibited some tumour preventative effect

Androgens potent from a sex hormone standpoint were effective agents in preventing the occurrence of expected adrenocortical tumours in gonadectomized strain CE mice

Steroids or steroid like substances which did not exhibit pronounced sex hormone activity as used may in general be considered weak or non effective adrenal tumour preventing agents in these experiments Increased absorption of sub stances with slight or moderate sex organ stimulating ability will increase their ability to prevent the occurrence of adreno cortical tumours and thus their secretory activity

Cortisone acetate and cortexone acetate were inactive in preventing tumour formation when administered in pellet form Houssay Higgins and Bennett (1951) have reported the latter to be effective when administered in oil

Pituitary removal was effective in preventing the adreno cortical tumours This has been more fully explored by Ferguson and Visscher (1953)

Purified pituitary hormones ACTH LH GH and FSH were administered to neonatally castrated female strain DBA mice Treatment was started when the mice were two months of age and continued by means of three injections daily at eight hour intervals until the animals were six months of age It was observed that the expected nodular hyperplasia of the adrenal cortices was not greatly modified by these treatments These experiments will be reported more fully at a later date

In our study of established adrenocortical tumours oestrogen was used because it had been the most effective agent found for preventing the occurrence of the expected tumours in gonadectomized mice Tumours were allowed to develop to nine months of age in the female and 12 months of age in the male and then treated for a three month period

and gonadal function that reacted most differentially on the mammary glands Upon transplantation of an adrenal tumour from one of the above cases the differential action on the mammary glands failed to appear, indicating the need for pituitary dysfunction for full secretory expression

We have also conducted a number of studies which attempt to modify the tumour occurring tendencies as well as the secretory activity of experimental adrenocortical tumours It will be recalled that without treatment nearly 100 per cent tumour incidence would be expected in gonadectomized strain CE mice when six months of age Since the tumours were obtained by gonad removal it would seem probable that substitution of gonad type hormones should reverse the tumour occurrence This was found to be true as is shown below Another approach to the problem of control was to depress the gonadotrophic functions of the pituitary or remove the pituitary itself Another possibility was to add pituitary hormones These possibilities were explored

The experimental strain CE mice were gonadectomized neonatally and then treated subcutaneously with various steroid and steroid like hormones in compressed pellet form, starting at two months of age and carried with continuous treatment to eight months of age at which time the animals were weighed autopsied and studied Thus the treatment was for a period of six months Gonadectomized control animals were observed without treatment to a similar age i e to eight months of age We have studied some 25 steroids some at as many as five dose levels

We now have evidence that various oestrogens are effective agents in preventing the occurrence of tumorous and cancerous changes in the cortex The exact structure of the oestrogen makes little difference except in the dosage requirement oestradiol oestrone oestriol equiline and the steroid like hormone stilboestrol (3 4 di *p* hydroxyphenylhex 3 ene) are all effective Oestriol was effective when absorbed from a pellet mixed 1 11 with cholesterol and with a calculated absorption of 0 0001 mg/day The synthetic oestrogen

frequently develop adrenal tumours but these lesions never attain the same size as those found in castrated untreated rodents No evidence was presented that the adrenocortical tumours produced unusual amounts of any hormone

Smith (1945) noted that the high breast cancer strain C3H mice when gonadectomized early in life began to have oestrual cycles between the fifth and seventh months at about the time when they developed hyperplasia of the adrenal cortices large cystic uteri and extensive development of the mammary ducts The low cancer (virgin) A strain mice on the other hand were still without oestrual cycles at the age of seven months The adrenals of the latter showed relatively little hyperplasia the uteri were small and the mammary glands were not strikingly enlarged

Casas (1954) observed that castrated strain C3H mice fed a stock diet of 0 3 per cent thiouracil developed adrenocortical and thyroid hyperplasia but never showed signs of the hormonal stimulation which is typical of the ovariectomized animal of this strain on stock diets

Bittner and his associates (1946 1954) called attention to a relationship between the mammary cancer inherited hormonal influence and inherited factors for adrenocortical tumour formation in gonadectomized mice The findings do not yet form the basis for a completely generalized theory

Through transplantation of adrenal tissue from different inbred lines into adrenalectomized gonadectomized F_1 hybrids Huseby and Bittner (1951) demonstrated that responsiveness of the adrenal tissue to gonadectomy is probably inherent in the adrenal tissue itself Our trans plantation studies are in accord with this hypothesis

Martinez and Bittner (1955) observed that the removal of one adrenal gland at the time of gonadectomy of female mice of strain C3H did not prevent the development of tumours in the remaining gland but most of the tumours did not show signs of the production of sex hormones

Casas King and Visscher (1949) found that 33 and 50 per cent caloric restriction in gonadectomized strain C3H female

with oestrogen in pellet form At the end of this period of treatment the tumours of these individuals were compared with those of the untreated controls Part of each group was allowed to continue another three months to test whether or not tumour growth would be re established after withdrawal of the oestrogen After this three months without treatment another set was treated for a further three month period in order to try to hold down tumour growth over a total nine month period Evidence was obtained that growth of an already established tumour of the carcinoma type could be inhibited greatly We were not able to eliminate completely the morphological change associated with the tumour Although the tumour pattern remained the cells within the tumour area became shrunken and the cells separated by connective and degenerative tissue Secretory activity as evidenced by failure to note androgenic changes in the treated tumour bearing mice was believed to have been effectively reduced

A number of reports from the extensive literature on experimental adrenocortical tumours and on their secretory activity will now be mentioned

Gardner (1941) reported adrenal tumours in a group of strain NH hybrid mice 40 to 65 days of age, following removal of their ovaries Fifteen of these mice were spayed when they were between 43 and 65 days of age Thirteen of these eventually developed adrenal tumours which were accompanied by signs of pronounced oestrogenic activity such as cornification of the vagina cystic glandular hyperplasia of the uterus and separation of the pubic bones

Dorfman and Gardner (1944) made a biological assay of spayed mice of the NH strain and found that ovariectomized mice bearing adrenocortical tumours secreted four times as much oestrogen in their urine and faeces as the intact females of the same strain They also reported that spayed mice with cortical tumours were more liable to have mammary cancer

Gardner (1947) reported that some strains of intact mice acquiring pituitary adenomas after treatment with oestrogens

attention to the fact that in man with unilateral adrenal tumour the adrenal without tumour is often markedly atrophic

In 1939 Speigel described the occurrence of adrenocortical adenomas in four guinea pigs castrated and then observed when 3½ to 4 years of age Stimulation of the accessory reproductive organs prostatic gland seminal vesicle and penis, in these male guinea pigs was described indicating androgenic hormones to be present

Keyes (1949) observed hyperplastic adrenal changes after gonadectomy of golden hamsters We have observed a number of male and female golden or Syrian hamsters gonadectomized at birth and autopsied at ages up to and beyond 20 months of age Adrenocortical tumours were present and accessory reproductive organs were indicative of the presence of oestrogenic and androgenic hormones It seems probable that the adrenocortical tumours were the source of these hormones in the gonadectomized hamsters (Woolley 1953) Franks and Chesterman (1956) described golden hamsters following prolonged treatment with stilboestrol Diffuse hyperplasia and tumour nodules were found in the adrenal glands Attention was called to the similarity of the total findings with those of Conn s syndrome in man

The studies concerned with secretory activity of adreno cortical tumours in man are not reviewed in this report It would seem to the author however that animal counterparts of the adrenal tumours and adrenal tumour syndromes of man have been observed and can now be reproduced experimentally for further detailed study

REFERENCES

ANDERSON W A D (1953) *In* Pathology 2nd ed p 1036 St Louis C V Mosby Company

BITTNER J J and FRANTZ M J (1954) *Cancer Res* 14 81

BITTNER J J and HUSEBY R A (1946) *Cancer Res* 6 235

CASAS C B (1954) *Fed Proc* 13 23

CASAS C B KING J T and VISSCHER M B (1949) *Amer J Physiol* 157 193

mice did not prevent adrenocortical hyperplasia formation but retarded, or prevented its gonadal function as judged by the lack of stimulation of secondary sex organs Dalton Edwards and Andervont (1943) described a spontaneous transplantable, adrenocortical tumour arising in a C strain mouse Major changes in the accessory sex organs were not associated with this tumour although probably small amounts of oestrogenic hormones were present

Adrenocortical neoplasms have been observed in the rat in several different laboratories These adrenocortical tumours have been associated with changes in the accessory reproductive organs indicative of sex hormone production presumably from the adrenal cortex Cortisone like hormones are also thought to be produced by these tumours Hertz (1950) called attention to the presence of adrenocortical tumours in castrated rats when observed at advanced ages There was evidence that these tumours produced sex like hormones Houssay and co workers (1953) found that adrenocortical tumours develop in white laboratory rats following gonadectomy but much more slowly than in certain strains of mice Many adenomas elaborate oestrogens one year after castration The oestrogenic secretion is controlled by pituitary gonadotrophins The appearance of these adrenal tumours is due to a disturbance of pituitary functions caused by an alteration of the pituitary gonadal balance Mulay and Eyestone (1955) describe adrenocortical adenocarcinomas in female rats of the Osborne Mendel strain, aged 18 months or older which were under observation for development of liver lesions A transplanted tumour caused reduction in size and weight of the adrenals of the host Microscopic examination of the adrenals showed that most of the loss in size could be attributed to atrophy of the adrenal cortex Four cases of adrenocortical tumour were unilateral and the contralateral adrenal was atrophic This is presumably mediated through suppression of ACTH by an adrenocortical hormone elaborated by the tumour No striking changes were observed in any other endocrine organs of the host Anderson (1953) calls

MARCHAND F (1891) *Int Beitr z wiss Med Festschr Virchow* 1 357
MARTINEZ C and BITTNER J J (1955) *Cancer Res* 15 612
McCOY G B (1909) *J med Res* 21 285
MULAY A S and EYESTONE W H (1955) *J nat Cancer Inst* 16 723
MURRAY W S (1927) *Science* 66 600
MURRAY W S (1928) *J Cancer Res* 12 18
SLYE M HOLMES H F and WELLS H G (1921) *J Cancer Res* 6 305
SMITH F W (1945) *Science* 101 279
SMITH F W and BITTNER J J (1945) *Cancer Res* 5 588
SMITH F W and BITTNER J J (1948) *Cancer Res* 8 641
SPEIGEL A (1939) *Virchows Arch* 305 367
WOOLLEY G W (1945) Josiah Macy Jr Foundation Trans 11th Conf Metabolic Aspects of Convalescence p 77
WOOLLEY G W (1946) Josiah Macy Jr Foundation Trans 13th Conf Metabolic Aspects of Convalescence p 111
WOOLLEY G W (1948) *Acta Un int Cancr* 6 265
WOOLLEY G W (1949) *Ann N Y Acad Sci* 50 616
WOOLLEY G W (1950) *Recent Progr Hormone Res* 5 383
WOOLLEY G W (1951) *In* Pituitary—Adrenal Function p 56 Baltimore Horn Schafer Co
WOOLLEY G W (1953) *Anat Rec* 115 381
WOOLLEY G W (1954*a*) *Proc 2nd nat Cancer Conf* 2 1509
WOOLLEY G W (1954*b*) *J nat Cancer Inst* 15 717
WOOLLEY G W and CHUTE R (1947) Josiah Macy Jr Foundation Trans 16th Conf Metabolic Aspects of Convalescence p 65
WOOLLEY G W and DICKIE M M (1947) *Cancer Res* 7 72
WOOLLEY G W DICKIE M M and LITTLE C C (1952) *Cancer Res* 12 142
WOOLLEY G W DICKIE M M and LITTLE C C (1953) *Cancer Res* 13 231
WOOLLEY G W FEKETE E and LITTLE C C (1939) *Proc nat Acad Sci Wash* 25 277
WOOLLEY G W FEKETE E and LITTLE C C (1940) *Proc Soc exp Biol N Y* 45 796
WOOLLEY G W FEKETE E and LITTLE C C (1941) *Endocrinology* 28 341
WOOLLEY G W FEKETE E and LITTLE C C (1943) *Science* 97 291
WOOLLEY G W and LITTLE C C (1945) *Cancer Res* 5 193
WOOLLEY G W and LITTLE C C (1945) *Cancer Res* 5 203
WOOLLEY G W and LITTLE C C (1945) *Cancer Res* 5 211
WOOLLEY G W and LITTLE C C (1945) *Cancer Res* 5 321
WOOLLEY G W and LITTLE C C (1945) *Cancer Res* 5 506
WOOLLEY G W and LITTLE C C (1946) *Proc nat Acad Sci Wash* 32 239
WOOLLEY G W and LITTLE C C (1946) *Cancer Res* 6 712
WOOLLEY P G and WHERRY W B (1911) *J med Res* 25 205

CHRISTY N P DICKIE M M ATKINSON W B and WOOLLEY G W (1951) *Cancer Res* 11, 413
CHRISTY N P DICKIE M M and WOOLLEY G W (1950) *Endocrinology* 47 129
CORI C F (1926) *J Cancer Res* 10 265
CORI C F (1927) *J exp Med* 45 983
CRECCIO L (1865) Il Morgani (Referred to by Marchand 1891)
CURTIS M R BULLOCK F D and DUNNING W F (1931) *Amer J Cancer* 15 67
DALTON J A EDWARDS J E and ANDERVONT H B (1943) *J nat Cancer Inst* 4 329
DICKIE M M (1948) *Acta Un int Cancr* 6 252
DICKIE M M and WOOLLEY G W (1949) *Cancer Res* 9 372
DORFMAN R I and GARDNER W U (1944) *Endocrinology* 34 421
DUNNING W F and CURTIS M R (1952) *Cancer Res* 12 702
DUNNING W F CURTIS M R and SEGALOFF A (1953) *Cancer Res* 13 147
FEKETE E and LITTLE C C (1945) *Cancer Res* 5 220
FEKETE E WOOLLEY G W and LITTLE C C (1941) *J exp Med* 74 1
FERGUSON D J and VISSCHER M B (1953) *Cancer Res* 13 405
FEODOSSIEW H W (1906) *Russk Vrach* 5 135
FRANKS L M and CHESTERMAN F C (1956) *Lancet* 2 1193
FRANTZ M (1948) *Anat Rec* 100 662
FRANTZ M J and KIRSCHBAUM A (1948) *Proc Soc exp Biol N Y* 69 357
FRANTZ M J and KIRSCHBAUM A (1949) *Cancer Res* 9 247
FRANTZ M J KIRSCHBAUM A and CASAS C B (1947) *Proc Soc exp Biol N Y* 66 645
GARDNER W U (1941) *Cancer Res* 1 632
GARDNER W U (1947) *Recent Progr Hormone Res* 1 217
HERTZ R (1950) *Recent Progr Hormone Res* 5 401
HOUSSAY B A CARDEZA A F HOUSSAY A B and PINTO R M (1951) *Rev Soc argent Biol* 27 315
HOUSSAY B A CARDEZA A F HOUSSAY A B and PINTO R M (1952) *C R Soc Biol Paris* 145 610
HOUSSAY A B HIGGINS G M and BENNETT W A (1951) *Cancer Res* 11 297
HOUSSAY B A HOUSSAY A B CARDEZA A F FOGLIA V G and PINTO R M (1953) *Acta physiol latinoamer* 3 125
HUSEBY R A and BITTNER J J (1950) *Cancer Res* 10 226
HUSEBY R A and BITTNER J J (1951) *Cancer Res* 11 954
KEYES P H (1949) *Endocrinology* 44 247
KING J T CASAS C B and CARR C J (1948) *Fed Proc* 7 64
KING J T CASAS C B and VISSCHER M B (1949) *Cancer Res* 9 436
KING J T VISSCHER M B and CASAS C B (1947) *Fed Proc* 6 14-
KIRSCHBAUM A FRANTZ M and WILLIAMS W L (1946) *Cancer Res* 6 707
LOEB L (1919) *J med Res* 40 47-

of mammary adenomas Lately there have appeared in the literature a few reports on elderly dogs with spontaneous diabetes At autopsy all these animals had gross nodular hypertrophy of the adrenals In one of these cases which we described the posterior pituitary showed a gross predominance of eosinophilic cells and this has also been found in other cases of spontaneous canine diabetes I am suggesting to Dr Furth therefore that a consequence of prolonged oestrogen deficiency might be a hypertrophy of eosinophil cells which would lead to hyperproduction of somatotrophic hormone This would exert an influence on the islets of Langerhans of the pancreas giving rise first to hyperactivity of the islets and then to exhaustion and diabetes It is very striking that spontaneous diabetes in dogs which is not rare at all seems to be part of a syndrome of age these dogs show atrophy of the ovaries (or the ovaries have been taken out) mammary adenomas gross nodular hypertrophy of the adrenal cortices and excess of eosinophilic cells in the pituitary One might even consider oestrogen deficiency as a contributing factor to the production of human diabetes by the mechanism which Dr Furth has described

Huseby The hypothesis that Dr Furth has outlined is probably correct in essence However exactly what hormones are involved is more difficult to ascertain Ferguson and Visscher (1953 *Cancer Res* 13 405) demonstrated that castrate hypophysectomized mice do not develop hyperplastic adrenals One might therefore assume that excessive stimulation by some pituitary trophic hormone is responsible for the development of the hyperplasia Houssay and co workers have shown that in rats the gonadotrophins are important in stimulating oestrogen production by adenomata developing after castration Recently Dr Segaloff and I worked with an oestrogen producing transplanted adrenocortical carcinoma that arose in a castrate CE mouse Oestrogen production was stopped immediately by hypophysectomy administration of ACTH did not result in a resumption of oestrogen production by this tumour but the administration of a mixed pituitary gonadotrophin preparation did Interestingly enough Martinez and co workers (1956 *Proc Amer Ass Cancer Res* 2 No 2 p 131) have presented data suggesting that the administration of ACTH inhibits oestrogen production by the hyperplastic adrenals of castrate ZBC mice Taschkis and co workers (1954 *Proc Soc exp Biol N Y* 85 422) demonstrated that sex steroid producing transplanted adrenocortical carcinomas that arose in castrate CE mice contained glucocorticoid activity as well but they were unable to demonstrate *in vitro* conversion of cortexone to compounds with glucocorticoid activity In connexion with experiments with testicular interstitial cell tumours we have administered a purified pituitary LH preparation to both castrate and intact BALB/c mice for periods up to four months and have not seen any tendency for this preparation to favour adrenocortical hyperplasia From all this work I think one must conclude that the relationship of the pituitary to the development of and subsequent hormone production by the altered adrenals of castrate rodents is a rather complex one

Woolley Houssay has published studies concerning adrenocortical

DISCUSSION

Furth May I sketch the pathogenic mechanism of the changes Dr Woolley has just described if only for the sake of discussion Removal of the gonads stimulates the gonadotropes of the pituitary (Step 1) The gonadotropes stimulate certain cells of the adrenal cortex bring ing about a functional metaplasia so that these cells are capable of secret ing gonadal hormones (Step 2) The gonadal hormones as Dr Woolley has shown include oestrogens and the oestrogens act as stimulants of the mammotropes (Step 3) which stimulate the mammary gland (Step 4) The pituitary tumours could be either gonadotrophic or mam motrophic *It follows that in animals hypophysectomized soon after* castration adrenal tumours fail to develop in those hypophysectomized after adrenal tumour development the mammary glands will not become hyperplastic

Dr Woolley spoke of basophilic pituitary tumours The mammo trope is eosinophilic and the delta cells or gonadotropes are basophilic Both types can conceivably be induced by castration early in life

We found in mice and rats adrenal tumours which unlike any of those described by Dr Woolley secrete corticoids These adrenocortical tumours were transplanted in series in isologous strains of rats and mice These are malignant tumours because they metastasize extensively in almost every host nevertheless they secrete corticoids and respond to ACTH The best index of such secretion is adrenocortical atrophy in tumour bearing hosts due to specific inhibition of ACTH Signs of mineralocorticoid secretion are tremendous intake of water polyuria and hypernatraemia Glucocorticoid secretion is indicated by leuco paenia and eosinopaenia (Cohen A I Furth J and Buffett R F (1957) *Amer J Path* 33 631)

Cohen incubated these tumours with ACTH and found an increase of Δ^4 3 ketosteroids in proportion to quantity of ACTH added This test *of in vitro* responsiveness to ACTH of corticoid tumours which *may be* done with a tumour slice of 50 to 100 mg is applicable to man (Cohen A I Bloch E and Celozzi E (1957) *Proc Soc exp Biol N Y* 95 304) It is remarkable that the adrenal tumour is more sensitive to ACTH *in vitro* than the normal rat adrenal Since 0 1–0 2 m u of ACTH is capable of stimulating these adrenal tumours this is perhaps the most sensitive specific ACTH assay Dr Bloch found that the major produce of the normal mouse and rat adrenal is corticosterone (about 70 per cent) and 11β hydroxy Δ^4 androstene 3 17 dione is next in quantity In adrenal tumour incubates there is relatively less cortico sterone and more *11β hydroxy Δ^4 androstene 3 17 dione*

Woolley Dr Furth mentioned the eosinophil cells of the pituitary We found that pituitary tumours characteristically originate in two locations As they begin to grow and develop we find the neighbouring eosinophils greatly enlarged We have not in our series seen the eosino phil cells taking an active part in forming a neoplasm

Groen In elderly dogs especially female dogs and especially female dogs that have been spayed one very frequently sees the development

CONSIDERATION OF SOME TYPES OF ADRENAL TUMOURS

ALICE M ROBINSON ANN DIMOLINE AND DORA G JONES
Pathology Department St Bartholomew s Hospital London

THE usefulness of the estimation of urinary 17 ketosteroids as an aid to clinical diagnosis is frequently debatable but there seems to be one condition in which it is generally held to have considerable value and that is in the differentiation between adrenal hyperplasia (not congenital) and adreno cortical tumours

Dorfman and Shipley (1956) give an excellent summary of the position in their study of the androgens In the cases of adult adrenal hyperplasia producing virilism elevated 17 ketosteroids may be encountered but where the clinical character is that of Cushing s syndrome the 17 ketosteroids are not usually elevated although the cortin like excretion is usually increased In the case of virilizing adrenocortical tumours the 17 ketosteroid excretion is frequently more dramatically elevated than in virilism due to hyperplasia and values may be well above 100 mg /day Metastatic malignant tumours are sometimes responsible for an excretion which exceeds 1 000 mg In Cushing s syndrome the 17 ketosteroids are usually low when the tumour is benign and frequently but not always excessive when the tumour is malignant

A further aid in the differentiation of adrenocortical tumour from hyperplasia is the estimation of the 3β hydroxy 17 ketosteroids In the case of adrenocortical hyperplasia the 3β hydroxy 17 ketosteroids are either of normal or only slightly elevated value but a striking difference may be seen in some cases of adrenocortical tumour where as much as 80 per cent of the total 17 ketosteroid may be in the β fraction which may represent as much as 500 times the normal output of β steroid

tumours in the rat I understand that he failed to get the tumours to full hormone production with gonadotrophic hormone alone in the hypophysectomized rat With the addition of ACTH he has interestingly enough caused them to secrete at an approximately normal level One might interpret these results in any of a number of ways there may be a structural building up of the cells so that gonadotrophins can function properly or perhaps production is helped on an endocrine level

Parkes I was very interested Dr Woolley in your remarks about the sex cycles in ovariectomized mice with adrenal tumours You said they mated at the proper time and I wondered what was the proper time in the sex cycle of mice without ovaries! Is it at the nucleated phase of the smear?

Woolley In general vaginal plugs were observed on days following oestrous like smears In the study we observed mice about 12 months of age and vaginal cycles were very irregular occurring at intervals of 1–24 days and oestrous cycles were variable in duration (1–11 days)

Parkes What is the mechanism for that cycle? Is the production of oestrogen by the tumour cyclic or is the sensitivity of the vagina cyclic?

Woolley As far as I know that is a question for the future

The procedure used in the preparation of these urinary extracts was acid hydrolysis followed by treatment with Girard's reagent and separation into the α and β fractions with digitonin

Normal Individuals

The values for a number of normal men and women obtained in our laboratory are shown in Table I

Table I

17 KETOSTEROIDS IN THE URINE OF NORMAL INDIVIDUALS

Sex	*Age*	*No of subjects*	*Total KS mg /day Mean*	*Total KS mg /day Range*	*3β KS mg /day Mean*	*3β KS mg /day Range*	*% 3β Mean*	*% 3β Range*
Men	20–48	13	15 7	(9 2–24 5)	2 1	(0 1–5 4)	13	(1–35)
Women	23–49	12	9 6	(6 6–15 9)	1 1	(0 1–[illegible] 7)	13	(2–22)

Idiopathic hirsutism

The values for a group of women where the symptoms were those of idiopathic hirsutism with or without menstrual irregularities are shown in Table II

Table II

17 KETOSTEROIDS IN IDIOPATHIC HIRSUTISM

Case No	*Age*	*17 KS mg /day*	*3β mg /day*	*% 3β*
H 1	27	10 3	1 2	11 5
H 2	25	27 3	2 1	7 5
H 3	20	23 4	3 2	13 4
H 4	19	2? 5	5 [illegible]	25 0
H 5	21	23 4	6 6	28 0
H 6	16	38 7	11 4	30 0

The 3β hydroxy 17 ketosteroids show a range from 7 5 per cent–30 per cent with a mean value of 19 per cent and an

Dorfman and Shipley (1956) record the 3β hydroxy 17 ketosteroids for 25 cases of adrenocortical tumour and find the β fraction varies from zero to 75 per cent and averages 45 per cent Only four of the cases were definitely in the normal range that is less than 20 per cent two of these cases were children who had benign tumours (Dorfman and Miller unpublished observations) and a third case was a Cushing s syndrome (Kepler and Mason, 1947)

Most of the cases described were suffering from malignant tumours but in many instances no statement of the nature of the tumour was available and Dorfman and Shipley found it impossible to draw any conclusion concerning the relationship between malignancy and the excretory level of this fraction, or to disclose any difference in excretion between tumours associated with virilism and those which resulted in Cushing's syndrome They conclude nevertheless that the assay of the β fraction may be of distinct help to the clinician in the differentiation of adrenocortical tumour from hyperplasia

We are in complete agreement with these authors as regards the value of the estimation of the 17 ketosteroid and the 3β hydroxy 17 ketosteroid as a diagnostic tool for adrenocortical tumours where these values are unduly elevated but we feel that the stress laid by some workers on this elevation as a positive test may lead to the danger of excluding the diagnosis of adrenocortical tumour where the 17 ketosteroids are only slightly elevated and the 3β output absent or in the lower level of the normal range We have met two cases of malignant adrenocortical tumours in adults where the β fraction has been less than 1 mg /day (less than 4 per cent) one a Cushing s syndrome and the other a mixed virilism and Cushing s

Seven cases of adrenocortical tumour are discussed which show variations ranging from the typical picture with a high 17 ketosteroid and a 3β content of 70 per cent to a case showing only a slightly elevated 17 ketosteroid and practically no 3β steroid but in which a high degree of malignancy was demonstrated

years had a history of 12 years amenorrhoea and 11 years hirsuties which had become worse during the last three years There were excessive amounts of hair on the face chest nipples arms and legs with a three year history of loss of scalp hair Other features were increase in weight (four years) and increasing thirst frequency and polyuria (two years) Blood pressure 190/130–200/140 The breasts were small and clitoris enlarged There was no osteoporosis There was a palpable mass in the left kidney region and a malignant suprarenal tumour was removed at operation The post operative blood pressure was 120/70 and her general condition apart from the hirsutism improved for a time but the patient died about 18 months later

The 17 ketosteroid excretion before adrenalectomy was 210 mg /day of which 87 mg (41 per cent) was 3β steroid and the water soluble reducing corticoids were 6 mg /day (normal values 2 mg) The 17 ketosteroid excretion fell after operation to 5 mg /day and rose during twelve months to 170 mg /day with a 3β fraction of 17 mg (10 per cent) The reducing corticoids fell to normal limits and then rose steadily in line with 17 ketosteroids to 6 mg /day

Case 3 A case of secondary adrenal carcinoma showing virilism was a 53 year old woman who had had a left nephrectomy performed ten months before from which she had never really recovered X ray examination and post mortem showed secondary deposits in the lungs liver vertebral bodies and left femur The 17 ketosteroid excretion was 57 mg /day and the 3β 17 ketosteroid fraction 10 mg (18 per cent)

Case 4 A 47 year old woman was another patient showing the mixed picture of Cushing s syndrome and virilism She had had two children who are now adult There was a 2–3 year history of obesity amenorrhoea and hirsuties there was no polydipsia or polyuria or glycosuria but a decreased tolerance to glucose Blood pressure 240/130 There were no purple striae X ray examination showed no significant abnormalities Bilateral perirenal insufflation showed a 5 cm

absolute excretion which varies from 1 2–11 4 mg per day with a mean value of 5 mg per day The mean percentage value is only elevated by 50 per cent on the normal mean value although the mean value for the absolute excretion in these cases represents an almost fivefold increase

Adrenocortical tumours

Of the seven cases to be described, five were patients in this hospital, the other two (Nos 2 and 7) were patients in St Mary s Hospital and we are indebted to Dr S L Simpson for these case histories and clinical material

Case 1 with a diagnosis of adrenocarcinoma, was a well built, friendly and intelligent child of 12½ years who during the preceding 12 months had developed a masculine voice marked hirsuties on the face and axillae with pubic hair to the umbilicus and fine hair on the chest back arms and legs She showed well marked facial acne and there were deposits of fat on the abdomen hips and buttocks but she was not unduly obese The breasts were adolescent but mostly fat and not mammary tissue The clitoris was markedly en larged and the menses had not commenced X ray examina tion of hands and ankles showed a bone age of about 14 years that is they suggest a slight precocity and there was no evidence of metastases in the skull long bones or chest but a slight general decalcification of the vertebrae

Laparotomy revealed the presence of a large tumour arising from the left suprarenal, depressing and possibly invading the left kidney There was a large mass about ten times the size of the suprarenal gland on the side of the right adrenal The tumour was inoperable and the patient died within a short time of leaving this hospital

The average 17 ketosteroid excretion was 240 mg /day of which 120 mg (50 per cent) was 3β hydroxysteroid

Case 2 was an interesting example of mixed adrenal hyper function showing symptoms of virilism and Cushing s syn drome A short stocky plethoric looking woman aged 40

It was decided to carry out a bilateral adrenalectomy to relieve the hypertensive symptoms but at operation a large adrenocortical carcinoma was found which was closely adherent to the aorta diaphragm pancreas and kidney It was not possible to remove the whole tumour although the left kidney was removed in addition

The larger portion of this tumour was a lobulated and partially encapsulated mass 10 5 × 9 5 cm weighing 265 g The smaller portion 7 × 3 5 × 2 5 weighing 25 g approximated in shape to a normal adrenal gland and was found to be entirely tumour tissue Histologically sections showed a well differentiated carcinoma of the adrenal cortex with abundant large sinusoidal vessels Sections of the kidney showed no evidence of malignant invasion the larger arteries were hypertrophied but arteriolar changes were inconspicuous

There was no great improvement in this patient s general condition after operation and no marked fall in 17 keto steroids which have remained at a level of 17–18 mg a day presumably a considerable degree of adrenocortical secretion continues This patient is being followed up at monthly intervals

Case 6 was a rather thin healthy looking girl of 18 years with a three year history of hirsutism and increasing oligomenorrhoea with complete amenorrhoea for two years marked hirsutism on the face nipples chest and limbs and masculine distribution of pubic hair There was hypertrophy of the clitoris and the breasts were somewhat atrophic There were no voice changes

X ray examination showed a normal chest Pre sacral insufflation combined with intravenous pyelogram and tomograms showed a rounded tumour in the right adrenal region measuring 7 4 cm in diameter At operation an adrenal tumour and the surrounding adrenal tissue on the right side were removed The tumour measured 7 × 7 × 5 cm and weighed 110 g It appeared to be well encapsulated and

circular shadow in the region of the right suprarenal gland and a well encapsulated tumour which was removed from this site was histologically a carcinoma

The urinary 17 ketosteroids before operation were about 47 mg /day with a negligible amount of β steroid, less than 1 mg /day and the reducing steroids were within the normal range

Among the general improvements resulting from the operation were a decrease in weight and hirsutism and a fall in blood pressure to 170/80 and of 17 ketosteroids to 5 mg /day This improvement was maintained for about 18 months when all the earlier symptoms returned some with even greater severity and the 17 ketosteroids rose to an average figure of 67 mg /day with 4 mg (17 per cent) of 3β hydroxysteroid

Deep X rays were given without any clinical improvement and at a second operation the right kidney and attached suprarenal tissues were removed but other secondary deposits were irremovable The patient died shortly afterwards

Histological examination of sections from the primary site showed that it was composed of islands of tumour cells separated from each other by large areas of necrosis and haemorrhage The growth was a very undifferentiated suprarenal carcinoma showing great pleomorphism and many nucleated cells Vines stain for the fuchsinophil granules was positive

Case 5 A 55 year old man with a Cushing s syndrome due to adrenocortical carcinoma was admitted to hospital on account of hypertensive heart failure He was plethoric and obese, the obesity was confined to face and trunk, there were no striae sugar tolerance was normal the 17 ketosteroid ex cretion was 28 mg /day with practically no β fraction, and the 17 hydroxycorticoids were 88 mg a day (normal values up to 25 mg) There was no indication of malignancy as the cause of the general ill health Intravenous pyelogram showed the left kidney displaced downwards and some sug gestion of an adrenal tumour above this

Table III

17 KETOSTEROIDS IN ADRENOCORTICAL TUMOURS

Case No	*Age*	*Sex*	*17 KS mg /day*	*3β mg /day*	*% 3β*	*Remarks*
PROVED MALIGNANCY						
1	17	F	240	120	50	Virilism
2	40	F	210	87	41	Cushing s +virilism
3	53	F	53	9	19	Virilism
4	47	F	47	1	2	Cushing s +virilism
5	55	M	28	1	4	Cushing s
NON MALIGNANCY						
6	18	F	300	[illegible]10	70	Virilism
7	30	F	6[illegible]	15	25	Virilism

A consideration of these tabulated results shows how difficult it is to draw conclusions relating malignancy to the 17 ketosteroid or 3β hydroxy 17 ketosteroid excretion or to observe any significant difference in excretion between the tumours associated with virilism and those which result in Cushing s syndrome

In the first group of proved malignancy the total 17 ketosteroids are all elevated for the age and sex from 28–240 mg /dav with a mean value of 122 mg /day

The range of the 3β hydroxysteroids is 1–120 mg /day and although two of the cases fall within normal limits the mean value for the group is 46 mg /day (23 per cent) A comparison of these values with those obtained in our normal subjects shows that the β percentage is not greatly elevated but that absolute excretion of 3β steroid is greatly increased

One of the practical difficulties which is associated with work of this nature is the impossibility of making an examina tion of urinary steroids of all the cases at comparable states of their disease In the results tabulated five cases were studied previous to any surgical interference and while both adrenals were present In cases 3 and 7 the examination was made within a few months of removal of the tumour of one adrenal

the surrounding adrenocortical tissue from which it was readily separated appeared healthy

Histological examination showed an adenoma of the adrenal cortex composed of well differentiated cells of fairly uniform type and no mitotic figures The only suggestion of malignancy was the presence of large sinusoidal capillaries with extremely thin walls which make the occurrence of blood borne metastases a possibility

The extremely high output of 17 ketosteroids which fluctuated considerably but which averaged about 300 mg / day with 210 mg (70 per cent) of the 3β steroids suggests that this tumour may be potentially malignant But the long history of onset the gross characteristics of the tumour which were benign and non invasive, and the abrupt fall of 17 ketosteroids to 12 5 mg on the day following the operation, settling to an average level of about 5 mg /day make it unlikely that any metastases from the tumour were present This patient continues to be well seven months after operation and her general condition is good but the facial hirsutism is persistent

Case 7 At the age of 20 years this patient presented a generalized hirsutism with amenorrhoea and obesity of the trunk and face Intravenous pyelogram showed an enlarged right adrenal At operation a tumour was removed together with the right adrenal After the operation she lost weight commenced menstruating and the 17 ketosteroids which were 143 mg /day prior to operation (estimated elsewhere) fell to 60 mg /day of which 15 mg (25 per cent) were 3β hydroxysteroids

This woman has continued well over a period of twenty years but her hirsuties has not varied during that time and it would seem that the tumour was non malignant but that the remaining suprarenal gland shows a considerable degree of hyperplasia

The steroid excretion in these seven cases is presented in Table III below —

considered to be 3β 17α dihydroxy Δ^5 pregnen 20 one This was confirmed by infrared analysis The second compound m p 198° (uncorrected) showed an unknown new absorption on infrared analysis the presence of the Δ^5 3β hydroxy group was definite but the position of the ketone groups uncertain It may possibly be 3β hydroxy Δ^5 pregnene 20 one or 11 20 dione, but confirmation awaits the isolation of this substance from other sources

Case 4 After purification by chromatography and crystallization this was shown by infrared analysis to have been a 50 50 mixture of dehydro*epi*androsterone and *epi*androsterone

Cases 2 *and* 7 It has not been possible to date to have effected really efficient separation of these cases but a consideration of the Zimmerman and Pettenkofer colours suggests that there is also a 50 50 mixture of *epi*androsterone and dehydro*epi*androsterone

Cortisone suppression and adrenocorticotrophic hormone stimulation

In only three of these cases was the effect of cortisone and ACTH on the steroid output investigated

Case 3 Cortisone 200 mg daily was given intramuscularly over ten days There was no significant fall in steroid excretion

Case 5 Administration of cortisol 25 mg daily for three days caused no suppression and adrenocorticotrophin zinc 40 units twice daily for two days did not give a significant increase

Case 6 The steroid output was not suppressed by 300 mg of cortisone daily for three days and the administration of adrenocorticotrophin zinc 80 units daily intramuscularly for three days gave an apparent increase the assessment of this patient is difficult owing to the considerable fluctuation in pretreatment levels

but we feel justified in including them in one class for the following reasons

In two other cases of proved malignancy (2 and 4) which were studied throughout the course of the disease although there was some variation in steroid distribution as shown in Table IV the total 17 ketosteroids which had fallen im

Table IV

17 Ketosteroids in early and terminal stages

Case No	*Age*	*Sex*	*17 KS mg/day*	*3β mg/day*	*% 3β*
2	40	F	210	87	41
			170	17	10
4	47	F	47	1	2
			67	4	17

mediately after the operation rose gradually during the terminal stages to settle at a new high level There was a change in the amount of the 3β steroid but nevertheless the total 17 ketosteroid in each case remained in the same range and the actual chemical nature of the 3β steroid was the same in both stages of the disease

Chemical nature of the 3β hydroxysteroid

These seven cases of adrenocortical tumours did not all excrete the same 3β hydroxysteroids

Cases 1 *and* 6 One was malignant and the other non malignant and they excreted dehydro*epi*androsterone only This was isolated and identified by classical chemical procedures and infrared analysis

Case 3 No dehydro*epi*androsterone or *epi*androsterone was isolated but two unusual 3β hydroxysteroids were obtained One of these steroids m p 250° (uncorrected) was from a consideration of its m p and a comparison of its chromogenic properties in the Zimmerman and Pettenkofer reactions

REFERENCES

DORFMAN R I and SHIPLEY R A (1956) Androgens Biochemistry Physiology and Clinical Significance New York John Wiley and Sons
KEPLER E J and MASON H L (1947) *J clin Endocrin* 7 543
SIMPSON S L (1950) *Post Grad med J* 29 184
SIMPSON S L (1951) *Bull N Y Acad Med* 27 723
SIMPSON S L (1953) *Prensa med argent* 40 799
SPENCE A W (1951) *Proc R Soc Med* 44 161
SPENCE A W (1952) *Proc R Soc Med* 45 161

DISCUSSION

Dorfman We entirely agree in principle with your results Dr Robinson It is a bit disappointing that after some 20 years of urinary steroid studies we cannot predict with certainty whether a patient has adrenal cancer It seems from the history of these cases that the majority that show high values of dehydro*epi*androsterone do have adrenal cancer but this is not invariably true

Pincus Is there any possibility that acid hydrolysis would give sufficient artifact to eliminate a significant amount of dehydro*epi*androsterone?

Robinson No we have done a lot of experiments on normal and on dummy urines and know the proportion in these I could put my values up about 40 per cent on the method as used in our laboratory but that would not make those 1 mg results up to anything like significant values I could give you masses of figures We also have some comparable experiments with enzyme hydrolysis but these do not put the values up appreciably either

Dorfman I think the other clinching argument is to be found in the cases of apparently non malignant tumours which Dr Robinson and others have found

Robinson By the time the next edition of your book comes out Dr Dorfman we may have followed that girl (case 6) sufficiently long and she may have a true malignancy but we are hopeful at the moment She went to her doctor at the age of 13 and he held the old fashioned view that you could not mess about with people s glands until they were 18 So she has a five year history of onset of the trouble and that makes it look as if it were not malignant

Crooke I have seen two cases in which there has been a remarkable change to malignancy One of them was in Cushing s original monograph Case 16 was a child of 12 who was admitted as a possible case of his syndrome though her symptoms were not absolutely clear-cut. She lived until she was 31 I think She had a remission for those 20 years and then she developed a highly malignant adrenocortical tumour with three month s history of rapidly increasing hirsutism and amenorrhoea The other case was a woman of about 3_ who had florid Cushing s syndrome followed by a complete spontaneous remission lasting for three

Conclusions

A summary of the levels of 17 ketosteroids and 3β hydroxy 17 ketosteroids in the three classes described, normals idiopathic hirsutism and adrenocortical tumours, is shown below

Table V

SUMMARY OF 17 KETOSTEROIDS

No of subjects	Total KS mg /day		3β KS mg /day		% 3β	
	Mean	Range	Mean	Range	Mean	Range
NORMAL ADULT WOMEN						
12	9 6	(6 6–15 9)	1 1	(0 1–2 7)	13	(2–22)
IDIOPATHIC HIRSUTISM						
6	24 0	(10 3–39)	5 0	(1 2–11 4)	19	([illegible] 5–30)
ADRENOCORTICAL TUMOURS						
7	140 0	(28–300)	6[illegible] 0	(1–210)	30	(2–70)

It is apparent that there is considerable overlap between the three classes although there is a definite upward gradation from normals to adrenocortical tumours for the mean values in all three groups, yet individual values which fall in one group could well fall in another class

Our general conclusion was stated at the beginning of this paper but may be re emphasized here

When the values obtained for the 17 ketosteroids and for the 3β hydroxy 17 ketosteroids are unduly elevated the probability that these are due to a malignant tumour is very great but the possibility of malignancy should not be excluded because the levels are normal or only very slightly elevated

Acknowledgements

We wish to acknowledge our indebtedness to the British Empire Cancer Campaign for generous grants to this hospital which have supported this work

Our thanks are also due to Dr Gallagher of the Sloan Kettering Institute for the infrared analysis and to Drs Cullinan Scowen S L Simpson and Spence for permission to use their case histories and for making available the clinical material

has the impression that these individuals respond more like people with the adrenogenital syndrome—in other words there is a relatively high response of the androgen components as compared to the corticoids Dr Mills and others have indicated the possibility that some of these idiopathic hirsutism patients may have mild cases of the adrenogenital syndrome

Luft Most people working in the field of clinical endocrinology have the problem of patients suffering from hypertrichosis Most of us know what a problem this is to the patients Since no effective treatment has been available so far we have tried a combined treatment with ethinyl oestradiol locally—only in the face—together with cortisone orally in a dose of 25–50 mg per day and in addition epilation by a trained person To our pleasure most of these patients have reacted favourably and the hypertrichosis has improved markedly or disappeared The treatment is given for about a year and in some simpler cases six months Severe cases may have to have electrolysis perhaps every second month after discontinuation of hormone administration many of them however have stopped having electrolysis The ethinyloestradiol is administered in 70 per cent alcohol in an amount of 10 mg per 100 ml Our control material consists of patients being treated for different lengths of time with epilation only

Crooke I think you are entirely right to use electrolysis but I am not so sure that it is necessary to give them any corticoids I think you get just the same results without

Luft As I mentioned we have a control group that has been treated by electrolysis only The electrolysis is much simpler when local oestrogen is used as well since it is easier and less painful

Finkelstein We had a very strange case of benign adrenal tumour We tried cortisone just to see what it was and instead of going down the ketosteroids went up four times from 16 to 60 mg on the third day and continued to increase for about a week So it was rather a steroid dependent tumour

Dorfman That is very remarkable

Pincus You and others have observed that these tumours were unresponsive to ACTH Dr Dorfman?

Dorfman That is generally true except that one of Dr Gallagher s malignant tumours I believe was responsive to ACTH and could be inhibited with cortisone Dr Furth s tumour too was a malignant adrenal tumour which was responsive to ACTH

Pincus I was wondering whether with this combination of high 17-ketosteroids lack of response to ACTH and lack of response to cortisone a method of differential diagnosis might be developed

Dorfman It is a hope but at the moment it seems rather difficult There are malignant tumours which may or may not respond to ACTH and malignant tumours which may or may not have high dehydroepiandrosterone

Robinson As a matter of fact we had a difference of opinion in the interpretation of the non malignant case I mentioned The registrar wanted to interpret it as positive but there was considerable fluctuation

years Then she developed a very malignant adrenocortical tumour with a short history of rapidly increasing hirsutism and amenorrhoea but no other symptoms of Cushing s syndrome Autopsies on both these cases have been reported previously Remissions and exacerbations are very typical of Cushing s syndrome Not many of them have prolonged remission but when they do I think we have got to be very much aware of the possibility of malignant changes supervening

Bolinger I can confirm that same observation We had a patient who died with a very malignant degree of tumour after a 20 year history of very florid masculinization and symptoms of carbohydrate disturbance It was apparently benign for 20 years but she finally died of adrenal malignancy Strangely enough her sister also had a masculinizing syndrome but we have not had the opportunity to examine her adrenals yet

Dorfman What about the hormonal analysis?

Bolinger It showed very high values for the 17 ketosteroids somewhat high values for Porter Silber and also a diabetic syndrome

Finkelstein Dr Robinson did the cases of idiopathic hirsutism respond to cortisone?

Robinson All the ketosteroids went down with cortisone but the hirsuties did not improve We can never touch their hairiness we can bring their periods back and we can make them more cheerful but they have to go to beauty specialists

Dorfman How long did you continue the cortisone treatment?

Robinson For a long time from six to 18 months and six or nine months after we stopped it their ketosteroids were down to absolutely minimal amounts about 7 mg per day and we seemed to have knocked them down for good Dr Spence could tell you a lot about these cases because he treated them all But there was not a trace of clinical improvement in the hirsutism

Dorfman In other words the rate of regrowth of facial hair for example was about the same as it had been before treatment

Robinson Yes I find that patients in the higher social classes do better than lower grade mentalities The people to whom it matters a lot go to a lot of trouble they have their electrolysis they shave and they use cosmetics to good effect but the low mentalities just go down and down

Finkelstein I think Dr Gallagher from the Sloan Kettering Institute reported that there were some differences in the ketosteroids and he could see an improvement in some patients

Robinson We have not yet had any who noticeably improved

Dorfman Have you done any ACTH stimulation studies on these patients? Possibly there may be preferential production of androgen as compared to corticoids

Robinson In one case only the 17 ketosteroids which were 26 mg / day rose to 62 mg on the first day and 92 mg on the second day after stimulation the 17 hydroxyketosteroids were 36 mg a day and rose to 71 and 73 mg /day respectively

Dorfman Dr Mills has done some studies of that sort and I think he

SOME STUDIES ON OVARIAN TUMORIGENESIS*

W U GARDNER
Yale University School of Medicine

THE qualities of neoplastic cells are as diverse as are their morphological types or possibly even more diverse The one characteristic quality that differentiates cancerous from normal tissues is the capacity to grow without restriction by those influences that limit proliferation of normal cells in the body Beyond this one characteristic of unrestricted growth and this too can be somewhat relative tumours may possess to different extents qualities of normal body tissues These normal qualities are probably not essential to the neoplastic attainment but exist because the tumour has retained some of the qualities of its tissue of origin

If the above generalization is reasonably tenable then the assumption might be made that the tumours retaining to the greatest extent the capacity to duplicate normal body function would be least malignant And this is probably usually true but not necessarily always so Trophoblastic tissue is invasive and in some species produces gonadotrophin To be sure the extent of invasion of trophoblast is limited in contrast to that of a chorionepithelioma which also retains its hormone producing qualities Because invasiveness is one of the qualities of normal trophoblast as well as of malignant tissues the production of gonadotrophin by a chorionepithelioma is not a contradiction to the above generalization

The hormone producing organs afford an interesting opportunity for the study of the retention of normal or accession of aberrant functions Hormones are usually

* The original observations reported in this paper have been supported in part by grants from the Anna Fuller Fund the Jane Coffin Childs Memorial Fund and by Grant C 343 National Cancer Institute U S Public Health Service

and if there is ketosteroid excretion of 300–400 mg a day the urine collection is extremely important and there might be errors in the ward So I find it very difficult to interpret the suppression or elevation when there is a fluctuating base line and I am not prepared to say it was definitely responsive

Dorfman The trouble is not necessarily with the endocrinology or the biochemistry The difficulty may be the definition of malignancy we try to bend everything to the definition

Furth Yesterday s discussion indicated that growth and secretion are not synonymous and I think there is ample evidence that invasiveness and secretory function often are not parallel to each other

mentioned above was one of the last of Dr Strong's EI strain

The first hormone producing tumour studied extensively in our laboratory was an ovarian carcinoma (Strong Gardner and Hill 1937) This tumour appeared in an untreated mouse of the CBA strain and was transplanted into mice of the same strain for a number of generations (Strong *et al* 1938) The tumour rarely grew in female hosts but grew in almost all male hosts (Table I) The mammary glands of the male

Table I

INFLUENCE OF SEX AND SEX HORMONES ON GROWTH OF A HORMONE PRODUCING TRANSPLANTED OVARIAN CARCINOMA THAT AROSE IN A CBA MOUSE. (SUMMARY OF DATA FROM STRONG *et al* 1938)

Strain	*No of Mice*	*Sex*	*Number Growing*	*Treatment*	*Growth Characteristics*
CBA	168	♀	8 (7 in first transfers)	None	Slow and delayed growth
CBA	153	♂	153	None	Progressive growth plateauing at 9–10 weeks
CBA × A	53	♀	10	None	As in CBA ♀
CBA × A	26	♂	26	None	As in CBA ♂
A × CBA	47	♀	9	None	As in CBA ♀
A × CBA	44	♂	44	None	As in CBA ♂
CBA	12	[illegible]	4	Castrate	Faster than in ♀
CBA	31	[illegible]	14	Castrate	Slow growth

hosts were enlarged as in oestrogen treated mice The tumour would grow in ovariectomized females would not grow in oestrogen treated hosts or actually regressed when oestrogens were injected after the tumour had started to grow These observations were among the first on experimental hormonal chemotherapy as well The injection of gonadotrophin gave somewhat equivocal results (Table II)

The above observations presented some interesting problems Here was an ovarian cancer—it metastasized occasionally—that produced oestrogen like effects and yet the presence of ovaries or of injected oestrogens inhibited the growth of the tumour The determination of hormone production was

produced in small amounts and by relatively small numbers of the body's cells They can be discerned usually in the body at relatively low molecular concentrations by the specific responses they evoke in the body Furthermore hormones may as regulators of rates or periodicities of body functions fluctuate from time to time and under the control of known or postulated mechanisms

A number of assumptions may be made 1 Tumours of endocrine glands may produce hormones comparable to those of the normal gland from which they were derived and hence produce responses comparable to those of a normal gland 2 Tumours may produce substances that are unusual to the *normal organ and that produce either the same or modified* responses of the hosts tissues 3 The production of hormone by a tissue may or may not be regulated by those mechanisms normally regulating the rate or controlling the periodicity of hormone production 4 The hormone production may be minute per unit of tumour tissue in contrast to the production by the normal organ but be evident because much larger masses of tissue are present or because hormone production is not restricted periodically in the tumour 5 Hormone production by a tumour may establish a series of hormonal imbalances that will elicit or evoke the appearance of other tumours

The writer was first impressed by hormone production by tumours in laboratory animals when he found in 1934 a pituitary chromophobe adenoma bilateral granulosa cell tumours multiple mammary cancers in well developed lactating mammary glands and a cystic hyperplastic uterus all in one animal (Gardner, Strong and Smith 1936) Which tumour came first? What if any causal relationship existed between the several tumours and abnormal *overgrowths?* The ovarian tumours were assumed to have appeared first the mammary and pituitary tumours consequently It took many years and interesting combinations of genetic factors and hormones before it was possible to re establish these tumour combinations probably because the "tumour mouse

granulosa tissues were transplanted subcutaneously only 42 per cent grew tumours 35 per cent of 694 female mice also grew transplanted tumours The percentage of successful grafts was as high as 54 per cent when the tumours were trans planted into the spleen The percentage of successful grafts was not much above the average even for those tumours that were carried for from 20 to 80 serial passages Hormone production was rarely detected until the tumour had attained a size of 1 cm or more Often the tumours did not attain palpable sizes for a number of months

The brief summary listed above indicates some of the difficulties of evaluation of much that has been written Too often experiments in which growth has not occurred have not been reported or the rate of increase in size of the tumours has not been mentioned But in the experience of Bali and Furth (1949) at least half of the animals did not provide growth of the tumour and then only a small number of them showed hormonal effects until the tumour had attained a diameter of 2 cm or more These points are emphasized here to impress the difficulties encountered in such investigations and the small amount of active hormones probably produced by each cellular unit or the small number of cellular units that may be producing hormone The experiences of other investigators would be not dissimilar to those mentioned above The possibility that the specific synthetic processes of cells of the tumour may be modified so that incomplete or modified steroids are produced of course cannot be ascertained by biological assays except under most unusual circumstances

The major studies in our laboratory have been on factors that influence the genesis of ovarian tumours and that may modify their subsequent growth and hormone producing characteristics

Factors influencing the origin of ovarian cancer

Ovarian tumours appear quite frequently in mice subjected to X ray (Furth and Butterworth 1936) or bearing intra splenic ovarian grafts (Li and Gardner 1947) Ovarian

Table II

INFLUENCE OF PITUITARY HORMONES AND PROGESTIN ON THE GROWTH OF A TRANSPLANTED OVARIAN CANCER IN CBA MICE FIVE MICE IN EACH GROUP (FROM STRONG *et al* 1938)

Treatment*	♀	♂	[illegible]	⚦
Pregnant Mare s Serum	+	0	0	0
FSH	0	+	0	0
Oestradiol Benzoate	—	—	0	0
Progestin		—		
Testosterone				0
Egg Albumin	0	0	0	0

+ Growth of tumour was augmented *0* no change in growth as compared with controls
— less growth than in controls

only qualitative Methods were not available to determine quantitative differences in hormone production in the different environments The total hormone production was probably quite low in contrast to the production by one small ovary The minimal amount of oestrogen required to inhibit the growth of the tumour was never determined but it seemed that the amount produced by intact ovaries was adequate Whether the oestrogen acted directly on the tumour or on the host s pituitary was not determined but because the tumour grew and produced oestrogen in male and gonadectomized mice it was indicated that the oestrogen acted on the pituitary gland

At about the same time it was noted that irradiated mice which developed ovarian granulosa cell tumours showed a higher percentage of mammary cancer than did mice similarly treated in which ovarian tumours did not appear (Furth and Butterworth 1936 Furth and Furth 1936) The tumours presumably produced some hormone that stimulated the mammary glands

The most extensive study of the growth of transplanted ovarian tumours of different types was done by Bali and Furth in 1949 These tumours appeared in mice that were irradiated many months earlier After they had grown to appreciable sizes they were transplanted into other mice of the same strain Of 759 male mice in which 7 different

produced after repeated or even a single transplantation This experiment was inadequate however in that all of the tumours were in the spleen and we may have been determining in part what was happening in the liver and not just in the tumour Inanition may although this seems improbable *have augmented the hepatic inactivation of oestrogens* Experiments by Drill and Pfeiffer in 1946 indicate that inanition reduces the oestrogen destroying capacity of the liver The more logical conclusion is that inanition greatly reduces the production of hormones by the tumour The livers of the mice on the reduced food intake weighed about two thirds as much as did those of the controls It is evident that tumours can form and grow under conditions that may be inadequate for the production of hormones and that hormone production is not an essential part of the biology of a glandular tumour but a retention of its normal function

Even under comparable conditions the histological types of tissues that appear in ovaries transplanted into the spleen differ greatly Five different types of tumour were noted in one study (Gardner 1955) Many tumours were not of a single histological type particularly those classified as type II (tubular adenomas) Evidence of hormone production was determined by observation of the uterus vagina pubic symphysis and mammary glands Fifteen of the 21 mice that showed no evidence of steroid hormone production had either coarse trabecular tumours (type I) or tubular adenomas (type II) Four mice had tumours of the massive type (IV) Most of the follicular and the fine trabecular tumours were associated with evidence of oestrogen production Thirteen of the 17 mice with coarse trabecular or massive type tumours were males In no instance did the males show evidence of androgen production *One female with a follicular type tumour* had a large clitoris and a fibrous uterine response indicative of androgen Progesterone as indicated by the response of the uterine stromal nuclei (Hooker and Forbes 1947) occurred in approximately half of the female mice showing oestrogen production and was not related to any one cellular type

cancers also occur in ovaries transplanted into the testes or less frequently subcutaneously in male mice (Gardner, 1957*a* and *b*) The tumours arose only in ovaries that had been subjected to damaging influences X rays rather selectively destroy ova and prevent the subsequent formation of new ova Few ova survive transplantation and those that regenerate replenish the population for a reduced period of time Tumorous growths occur in these depleted ovaries and sometimes endocrine functions are partially restored

The tumours arise in an environment presumably somewhat higher than normal in gonadotrophins The levels of gonadotrophin have been modified by hepatic inactivation of ovarian hormones produced in the intrasplenic grafts in gonadectomized animals (Biskind and Biskind, 1944, Li and Gardner 1947 Miller and Pfeiffer, 1950) by reducing the hormone produced by the gonad by a method such as irradiation (Vermande Van Eck and Chang 1955) by placing intact animals in parabiosis with castrated partners (Muhlbock 1954) or presumably by subjecting an ovary to the influences of a male host s gonadotrophin (Gardner 1957*c*)

Conditions which reduced the production of gonadotrophin reduced the incidence of ovarian tumours (Miller and Gardner 1954) Sixteen of 21 mice fed *ad libitum* acquired ovarian tumours in intrasplenic ovarian grafts whereas only five of 12 mice fed a calorically restricted ration and only three of 20 mice made hyperthyroid acquired them None of the tumours that appeared in the mice on the inadequate diet were associated with evidence of hormone production Most of the tumours in the mice given thiouracil (10 out of 21 mice) were associated with evidence of oestrogen or both oestrogen and progesterone production

This experiment however, was of special value because the spontaneous or original tumours were being studied The changes occurring in tumour cells after transplantation may be no less great than those that occur in normal cells subjected to transplantation The hormones produced by the tumour in its original host may not be the same as those

animals This indicated that the effects on the tumours and on whatever the non tumorous ovaries or adrenal glands were doing were similarly influenced by FSH The FSH seemed to increase the production of progestin and decrease the production of oestrogen The latter of course could not be ascertained critically by the methods used

Although the number and size of the tumours in the FSH treated mice are greater than in the controls the data are not sufficiently abundant to be conclusive

Injected FSH failed to modify the rate of formation of ovarian tumours in irradiated ovaries and probably the rate of growth of such tumours Nevertheless it did seem to modify the production of hormones by the tumour as well as by the non tumorous irradiated ovaries Several other groups of mice were given FSH for prolonged periods Mice of some groups were irradiated (175 r total body irradiation) and others were not Comparable animals were retained for controls

Five mice of the AC and CC stocks were irradiated when 63 to 76 days old Vaginal smears were taken during the subsequent 371 days (Table V) The mice of the two irradiated

Table V

THE EFFECT OF IRRADIATION ON THE OESTROUS CYCLES OF HYBRID MICE DURING THE FIRST 371 DAYS AND THE CYCLES OF UNTREATED CONTROL MICE DURING COMPARABLE PERIODS

	Age irradiated and smear started	*Age smears started*	*No of cycles/ days++**	*Age killed*	*Ovarian tumours histological type*
25 CC2	76		49/125	590	III
26 CC2	64		20/46	694	I
27 CC2	64		9/21	626	II
28 AC2	63		13/[illegible]9	693	II
20 AC2	63		4/10	435	—
30 AC2		52	52/158†	39[illegible]	
31 AC2		68	68/157	567	
32 CC1		[illegible]0	70/155	692	
33 CC1		71	71/151	692	
34 AC1		69	66/166	695	

Number of periods of cornified smears number of day that cornified smears predominated
† Died t 395 d y so only 343 d ys of vagin l smears were follow d

Table III

TREATMENT AND OBSERVATIONS ON IRRADIATED UNILATERALLY OVARIECTOMIZED HYBRID AND INBRED MICE GIVEN FOLLICLE STIMULATING HORMONE (FSH)

Group	No of Mice	Age at irradiation Average	Age at operation Average	Age when FSH started Average	Period treated Range	Period treated Average	Age at death Range	Age at death Average	Ovarian tumours No	Ovarian tumours Type†
A7	4	78	93	628	106–212	163	661–894	803	1	II
CC	2	56	125	553	107–151	129	659–704	682	2	II‡
CB	6	66	111	512	176–208	195	660–799	741	2	II
									3	I
									1	V
C57	5	99	130	543	9–212	104	529–826	656	1	II

The hybrid groups were derived as follows A7=A×C57 strain CC=CBA×C57 strain CB=BC×C57 strain C57 refers to the subline of mice carried in this laboratory for the past 14 years

† Refers to the types of granulosa cell tumours arising in BC and CB mice (Gardner 1955) I is a coarse trabecular type of granulosa cell tumour II is a tubular adenoma and V is a fine trabecular type of granulosa cell tumour

‡ One tumour measured 18× mm. and at necropsy was necrotic cell type was not determined

10 mm in diameter and two exceeded 5 mm (Table IV) The genital tissues of the mice with the four larger tumours indicated some oestrogenic response but no well defined progestin response

Vaginal cornification was followed through smears taken for periods of 25 and 50 days Cornified cells disappeared from the vaginae of the FSH treated mice both with and without tumours at an earlier age than in the untreated

Table IV

OBSERVATIONS ON IRRADIATED UNILATERALLY OVARIECTOMIZED HYBRID AND INBRED MICE SERVING AS CONTROLS FOR ANIMALS LISTED IN TABLE I

Group*	No of mice	Age at irradiation Average	Age at operation Average	Age at death Range	Age at death Mean	Ovarian tumours No	Ovarian tumours Type†
A7	5	71	85	661–848	775	1	V
CC	3	56	125	595–814	704	1	II
CB	5	59	102	500–857	705	2	I
						3	II
C57	5	101	133	644–844	776	2	II

* See Table III † See Table III

developed The one irradiated mouse without an ovarian tumour had an atrophic uterus

Twenty four mice of four different stocks were irradiated and vaginal smears were taken daily for 72 days (Tables VI VII) Half of the mice were given one Armour unit of

Table VII

EFFECT OF RÖNTGEN IRRADIATION ON THE SUBSEQUENT APPEARANCE OF VAGINAL CORNIFICATION OF MICE DURING TWO PERIODS—THE FIRST 72 DAYS AFTER IRRADIATION AND THE SUBSEQUENT 102 DAYS—AND ON OVARIAN TUMOURS

	Age irradiated	*No of cycles/ days++ during first 72 days after irradiation*	*No of cycles/ days++ during subsequent 102 days*	*Age killed*	*Ovarian tumours histological type*
7 CC	78	10/26	2/3	776*	
8 CC	78	7/12	6/11	776	
9 A7	89	7/12	3/6	724	II IV very small
10 A7	89	11/32	11/21	696	
11 A7	89	8/20	9/18	434	
12 A7	89	9/17	4/9	737	II V
19 CC	78	8/16	2/2	391	II V
20 CC	76	10/21	2/3	615	
21 A7	76	8/24	12/28	427	luteoma
22 A7	76	10/20	2/2	385	II luteoma
23 A7	76	10/26	6/19	546	V
24 CB	83	6/13	5/13	782	II

Left ovary removed at 185 days.

FSH every second day for the remainder of their lives and vaginal smears were taken daily for up to 102 days from all 24 mice The mice given FSH showed an almost immediate cessation of oestrous cycles (Table VI) Only one of the FSH treated mice had an ovarian tumour at death whereas seven of the untreated mice had ovarian tumours Again this difference is probably not significant It may be significant that two of these tumours were luteomas

groups showed an average of 24 and 8 periods of cornified vaginal smears The unirradiated mice of comparable groups during comparable periods had 71 and 62 cycles and 153 and 161 days of cornified smears Irradiation at this age usually reduced the number of cyclic periods of vaginal cornification

Table VI

Effects of röntgen irradiation* and subsequent injection of FSH on vaginal cornification and ovarian tumours in mice

	Age irradiated	*No of cycles/ No of days++ during 1st 72 days after irradiation*	*No of days of smears after FSH*	*No of cycles/ No of days++*	*Age killed*
1 CC2	74	6/18	102	0/0†	5"6
2 CC2‡	74	9/26	102	1/3	624
3 CC2	74	9/23	21	0/0†	167
4 CC2	74	8/21	102	0/0	572
5 CC1	78	9/24	102	1/1†	280
6 CC1	78	10/18	10.	0/0	686
		average 8 5/21 7			
13 BC	77	4/7	88	0/0	685
14 BC	72	4/12	102	0/0	274
15 BC	72	7/26	102	1/3	532
16 BC	72	6/13	48	1/1	19?
17 BC	72	7/17	102	0/0	680
18 BC	72	8/20	102	1/1	280
		average 6/16			

185 r total body irradiation (50 kv 15 ma filters 1/2 mm. Cu 1 mm. Al T S D — 45 cm.—rate of dose 75 r /min)
† 1 CC had the right ovary removed at the time FSH started and the second ovary 11 days later 3 CC2 h d the second ovary removed 21 days later and 5 CC1 had the second ovary removed 130 days lat r
‡ 2 CC had a small type V ovari n tumour

within a period of two months Vaginal cornification sometimes recurred in the latter part of the first year The differences in number of days of cornified smears and number of cycles were great in the different irradiated mice and quite uniform in the unirradiated controls (Table V) Four of the five irradiated mice had ovarian tumours when killed at 590 and 694 days of age The genital tissues of the irradiated mice with ovarian tumours and of the controls were well

depend in part upon pituitary hormones but that growth of the tumours may not be so dependent Whether these relationships will be consistent must await further experimentation

The great variation in the growth of transplanted ovarian tumours is interesting Some ovarian tumours when transplanted into related hosts appear as palpable growths within a few days and grow progressively to attain large sizes within a month Other tumours do not attain palpable sizes for many months and thereafter may grow quite slowly The slow growing tumours in general are those that produce the most striking endocrine effects

Several ovarian granulosa cell tumours grow more frequently and more rapidly in male hosts than in female hosts—especially those that grow more slowly and that are influenced by hypophysectomy The data available at this time are inadequate to permit correlation with structure or quality of hormone production The environment provided by the male seems to be particularly favourable to ovarian tumorigenesis as well As mentioned earlier ovarian tumours appeared frequently when ovaries were transplanted into the testes of mice Irradiated mice acquire ovarian tumours even when testosterone propionate is injected (Gardner 1950) in amounts adequate to prevent any increase in gonadotrophin that could be determined (Vermande Van Eck and Chang 1955) The male seems to favour ovarian tumorigenesis But is this true? A tumour may form but if the environment does not favour its growth—the reduplication of cells of its type—its presence may never be apparent We cannot be certain at this time whether we are concerned with the processes that are involved in the initiation of abnormal cells or in processes involved in the replication of these cells The writer would guess at this time that the latter may be the more important

General summary

Several types of experimentation indicate that ovarian tumours may be evoked in an environment of abnormal

Five unirradiated mice of the BC strain were given one Armour unit of FSH every second day for 194 days (Table VIII) During the course of treatment one or both ovaries were removed from each mouse for histological study to determine the effect of prolonged injection of the exogenous hormone Within 40 days the ovaries were composed almost entirely of old corpora lutea luteinized follicles and luteinized stroma *Very few follicles with small antra were present* and the number of primary follicles was reduced Anovular follicles were forming from the germinal epithelium Very

Table VIII

EFFECT OF FSH ON OESTROUS CYCLES OF NON IRRADIATED MICE

	Age smears started	*Age FSH started*	*Age first ovary removed*	*Age second ovary removed*	*Age killed*
1 BC	70	75	119	191	524
2 BC	70	75	119	254	652
3 BC	70	75	132	380	652
4 BC	70	75	132	579	579
5 BC	70	75	162	621	621

None of the mice showed vaginal cornification for the first two months after the beginning of FSH injections Cornified smears appeared only 4 to 13 days among the mice given FSH

few normal follicles remained after 75 days or more and after about 150 days anovular follicles were quite numerous The abnormal balance of gonadotrophin affected either the survival or the formation of ova or both The luteinized condition of the stroma was comparable to that described by Pfeiffer and Hooker (1942) as occurring in mice given PMS for long periods The mice had very few oestrous cycles after injection of FSH

The injection of exogenous gonadotrophin has not been studied extensively in mice bearing transplanted tumours to determine the effect upon either the rate of growth of the tumours or the production of hormones by the tumours The *effects of hypophysectomy* have likewise not been studied extensively A number of studies in our laboratory indicate that the hormone production by ovarian tumours may

REFERENCES

BALI T and FURTH J (1949) *Cancer Res* 9 449
BISKIND M S and BISKIND G R (1944) *Proc Soc exp Biol N Y* 55 176
BISKIND G R BERNSTEIN D E and GOSPE S M (1953) *Cancer Res* 13, 216
DRILL V A and PFEIFFER C A (1946) *Endocrinology* 38 300
ELY C A (1956) *Endocrinology* 59 38
FURTH J and BUTTERWORTH J S (1936) *Amer J Cancer* 28 66
FURTH J and FURTH O B (1936) *Amer J Cancer* 28 54
GARDNER W U (1948) *Cancer Res* 8 357
GARDNER W U (1950) *Proc Soc exp Biol N Y* 75 434
GARDNER W U (1955) *Cancer Res* 15 109
GARDNER W U (1956a) *In* Hormones and Carcinogenesis p 207 New York Academic Press Inc
GARDNER W U (1957b) In preparation
GARDNER W U (1957c) In preparation
GARDNER W U STRONG L C and SMITH G M (1936) *Amer J Cancer* 26 341
HOOKER C W and FORBES T R (1947) *Endocrinology* 41, 158
LI M H and GARDNER W U (1947) *Cancer Res* 7 549
MILLER O J and GARDNER W U (1954) *Cancer Res* 14 220
MILLER O J and PFEIFFER C A (1950) *Proc Soc exp Biol N Y* 75 178
MUHLBOCK O (1954) *Acta Un int Cancr* 10 141
PFEIFFER C A and HOOKER C W (1942) *Anat Rec* 84 311
STRONG L C GARDNER W U and HILL R T (1937) *Endocrinology* 21 268
STRONG L C HILL R T PFEIFFER C A and GARDNER W U (1938) *Genetics* 23 585
VERMANDE VAN ECK G J and CHANG C H (1955) *Cancer Res* 15 280

DISCUSSION

Woolley Have you any observations on differential staining of the pituitary during these tumorigenic periods to see what could be happening during this presumably high gonadotrophic activity?

Gardner No we have no significant observations on that Likewise the biological experiments upon which I would place rather greater weight have not provided evidence for added conviction of the accuracy of those interpretations shown on our diagrams We have not really proved our points adequately or completely

Muhlbock I would like to hear your comments on the male environment

Gardner A good many of the old experiments which I think were probably accurately done and wisely interpreted indicate that in the male there is a relatively greater amount of FSH than LH I assume

quantities or qualities or persistences of intrinsic gonadotrophins The injection of exogenous gonadotrophins has so far never been associated with augmented ovarian tumorigenesis with but one exception when it seemed to reduce the age of appearance of granulosa cell tumours in intrasplenic grafts The gonadotrophin most likely to be present in unusual amounts or for unusual periods seems to be FSH but the injection of FSH did not significantly modify the incidence, or reduce the age of appearance or rate of growth, of ovarian tumours

FSH when injected at the rate of one Armour unit every second day into normal mice, irradiated mice or mice with ovarian tumours markedly reduced and often almost prevented oestrous cycles as determined by vaginal cornification The ovarian follicles and stroma were composed of lutein like cells Many mice so treated showed evidence of progestin production Some ovarian tumours seem to depend upon gonadotrophin for hormone production as do normal ovarian tissues Further work must be done in this area however

A variety of histological types of tumours may be associated with oestrogen production Evaluations of this type are treacherous though because few primary ovarian tumours consist exclusively of one type of cell The rate of hormone production in physiological units by tumours must be quite small per cell unit in contrast to that of non tumorous tissues

A hypothesis was proposed that ovarian tumorigenesis occurs because of marked damage to the ovary such as is attained during irradiation or transplantation but that in certain environments the proliferation of deviated cells does not occur This would account for the high incidence of ovarian cancer in ovaries transplanted into the testes and the failure of testosterone propionate to prevent ovarian tumours in irradiated mice The possibility exists that the influences affecting the initiation growth and function of ovarian tumours may all be different

is it that even though you can induce them in the rat by the Biskind technique you cannot induce them in the rat by total body ionizing irradiation? Nor were they induced in women even though tens of thousands of them have been irradiated to cause sterility or fertility In mice irradiation of the ovaries alone is adequate to produce ovarian tumours

Finally I should like to comment on cell type and hormones produced Transplantation studies disclosed two functional cell types granulosa cells causing changes indicative of oestrogen production and luteomas suggestive of progesterone production with unexplained androgenic and corticoid effects This scheme is not generally accepted and there is much debate as to the functions of the theca cells

Gardner To be very brief and as accurate as possible I think there is no relationship between cell type and function I have seen some of the most outstanding masculinization from a follicular type of granulosa cell tumour I would say that in general the luteoma tumours are more frequently associated with androgen production than are the granulosa cell type but this is not consistently so Some of our experiences with the luteomas and the granulosa cell tumours are just exactly the reverse of yours We formerly saw a number of luteomas and now we get only a few I do not know why that is

Dorfman I believe in one of your experiments there was an interesting progression Prof Gardner At an early stage you had what appeared to be the full complement of sex hormone biosynthesis progesterone androgen and oestrogen Then you had a loss in oestrogen biosynthesis with androgens still being produced and then eventually the androgen biosynthesis dropped out and you were left with glands which only produced progesterone When oestrogens are produced you have the full complement of steroids produced and as these tumours progress they become more primitive you might say and lose certain enzyme systems going back further until perhaps they produce only progesterone or eventually no hormone whatsoever Some correlation might be made with the cell types as you say and this scheme (cholesterol→ progesterone→ androgens→ oestrogens)

Huseby According to this scheme it would not bother you if you found a tumour that produced biologically detectable amounts of oestrogen without producing biologically detectable amounts of androgen since the activity of oestrogens is so much greater on a molar basis

Dorfman That is correct and it is entirely possible that the distribution of any one of the single components may vary depending upon the speed and regulation But I would suspect that if you found oestrogen and if you tried hard enough you would demonstrate at least minute amounts of the other steroid hormones

Gardner One important thing is that this particular sequence occurs in the non tumorous ovary under chronic stimulation with gonadotrophins This occurred in animals that were given pregnant mare serum for periods up to 455 days they did not develop tumours but they developed these massive luteinized ovaries and we have not been able to reproduce similar changes in any tumour so far

that in these animals there is relatively higher FSH in contrast to LH in the internal environment We can also demonstrate this in another type of experiment which has not been quite completed in our laboratory We can change the sex nature of the pituitary as Dr Pfeiffer showed many years ago by injecting testosterone during the first two weeks of life If we do that transplanted or intact ovaries respond as do transplanted ovaries in the males and there is an apparently permanent pituitary modification towards the male type So what I mean by the male environment (and this is hypothetical because it has not been proved in this particular experiment) is an environment with relatively more FSH than LH and presumably similar to that in the male

Leathem Regarding the influence of steroids in early life it may be pertinent to remind you that we find that a single injection of testosterone propionate at five days of age will permanently sterilize a female mouse and if we administer oestradiol as a single injection at five days of age we also permanently sterilize these mice However we find that administration of testosterone propionate results in these animals going into constant oestrus and we had some which were in oestrus for periods of 40 to 50 days During that time they refuse the male In contrast the sterilized animals with the oestrogen are essentially in constant dioestrus and though we end up with an ovarian picture which shows that they contain follicles they fail to ovulate and they fail to form corpora lutea This treatment certainly disturbs the initial pituitary efforts to create a normal balance at five days of age and yet the type of secretion given them at this age has quite a different effect on the eventual phenomenon as estimated by cycles

Furth I should like to supplement what you have said Prof Gardner Since 1930 I have so to speak lived with these ovarian tumours They crop up inevitably in various radiation studies in over 50 per cent of irradiated females Most of your description relates to granulosa tumours Most induced tumours are complex but by selection one can obtain pure cell lines The granulosa has been studied by many investigators but I do not understand why the luteomas were neglected This is an entirely different and fascinating tumour The granulosa tumours are oestrogenic The luteomas have not only progestational but also androgenic effects with profound atrophy of the adrenal cortex We should now like to reisolate them Originally we had many granulosa tumours now most of our radiation induced tumours are luteomas What determines whether the tumour will be a luteoma or a granulosa? What is the role of FSH and LH in their induction and why are they often mixed? These tumours are ideal subjects for biosynthetic studies

Next I should like to comment on the role of ionizing irradiation in inducing these tumours At first I thought they were due to direct hit mutations in line with the prevalent concept in the thirties In our present concept ionizing irradiation alters cells in the way Prof Boyland mentioned yesterday so that their responsiveness is changed consequently the feedback mechanism is disturbed This is a powerful preparatory change to tumour development Ovarian tumours did not appear in women in Japan exposed to total body irradiation Why

BIOCHEMISTRY OF CYSTIC OVARIES*

JAMES H LEATHEM

Bureau of Biological Research Rutgers University New Brunswick

ONE of the most common causes of infertility and associated reproductive disorders encountered in medicine and in veterinary practice is the cystic ovary Follicular cysts are overgrown or persistent Graafian follicles generally devoid of luteinization Lutein cysts are also formed from unruptured follicles Both types represent a failure of ovulation and a disruption of the normal cyclic pattern of the female

Spontaneous ovarian cysts and tumours have been noted in the human being as early as the second month of life and in all periods of sexual maturity (Speert 1949) Follicular cysts may be bilateral and multiple Although the neoplastic potential of the ovarian cyst is fairly low survival in estab lished malignancy of the ovary is discouragingly poor (West 1955)

Cystic ovaries are an important cause of reproductive disorders in domestic animals Irregular oestrous cycles infertility and nymphomania have been associated with functional and non functional cysts in the cow (Casida Casida and Chapman 1951) Nalbandov (1952) believes cystic ovaries to be the second most common cause of infer tility in swine

The factors causing the development of ovarian cysts are ill understood but the delicate balance which exists between the hypophysis and the gonad is involved Factors which influence the hypophysial gonadal axis therefore could induce abnormal ovaries However these factors are not necessarily readily apparent as polycystic ovaries may exist

* This investigation was supported by a research grant (RG-4451) from the National Institutes of Health U S Public Health Service

Parkes Where do these granulated cell tumours in the irradiated ovary come from?

Gardner Dr Furth mentioned that he and I apparently hold the minority opinion that they arise in relationship to the tubular ingrowths from the germinal epithelium

Parkes In what sense are they granulosa cell tumours?

Gardner Well where does the granulosa cell come from originally? I think we can get into a very nice embryological discussion here

Woolley What about the place of atretic follicles in this picture? Do they produce tumours?

Gardner The follicles have disappeared long before indications of tumour formation An important point here is that we do not know the exact time at which the original neoplastic change takes place We really are concerned with three things (1) The time at which the neoplastic change takes place (2) The time at which the neoplastic cell acquires an environment that will favour its growth if the neoplastic cell is formed but if its environment does not favour its growth we do not know of the change (3) Then in the endocrine experiments we want to know about the environment that will maintain the hormone producing capacity of the cell The neoplastic change might take place as soon as the first tubular epithelial ingrowth appears it might take place as soon as the very small anovular follicular structure is formed but how one can tell that I have no idea

Parkes That was the thing that was worrying me because by the time that irradiation is over and everything has settled down there is nothing very much left of the ovary in the case of the mouse There is nothing visibly identifiable as granulosa

Ovarian secretion of oestrogen and progesterone is well known Chorionic gonadotrophin caused secretion of oestrogen from the immature rat ovary detectable in circulation 26 hours after the first injection (Zondek and Sklow, 1942) Androgen secretion by the ovary can be detected in normal rats and becomes enhanced by transplanting the ovary to the ear (Parkes 1950) and to the spleen (Kullander 1956) In the adrenalectomized rat survival is extended by administration of pregnant mare serum gonadotrophin

There is a paucity of information concerning the chemistry or function of ovarian cysts and tumours *Follicular cysts* are generally associated with oestrogen secretion (Garm 1949) as are granulosa cell tumours (Crelin and Wolstenholme 1951) Furthermore granulosa cell tumour transplants in mice respond to pregnant mare serum with an increased oestrogen release (Green 1956) Nevertheless more information is needed on steroid chemistry for despite oestrogen formation the endometrium is not always fully developed in the presence of a granulosa cell tumour The polycystic Stein Leventhal ovary may secrete progesterone (Fisher and Riley 1952) as does the lutein cyst (Nalbandov 1952) with clitoral enlargement noted in the presence of lutein cysts in sows Pregnant mare serum will induce multilocular cysts in the immature cat from which oestrogen is secreted but luteinization may be observed in 10 to 20 days with the secretion of progesterone (Starkey and Leathem 1943)

Arrhenoblastomas and lipoid cell tumours elaborate androgen Urinary steroids in a patient with a luteoma revealed an increase in androsterone (Engel Dorfman and Abarbanel 1953) Watts and Adair (1944*a*) assessed the oestrogen in cyst fluid and obtained a positive response in 86 per cent and a titre of 0 07–33 rat units (r u) per ml Forty six per cent of lutein cysts contained oestrogen in amounts from 0 12–2 r u per ml whereas only three of 55 malignant cysts contained oestrogen Further complication is added by the failure to be able to correlate morphology of ovarian tumours and quality of secretion following transplantation (Green, 1957)

even though follicle stimulating hormone levels are normal (Kleinfelter and Seeger Jones, 1954) *Nevertheless*, transplantation of ovaries to the spleen in gonadectomized mice and rats induced ovarian tumours, possibly by altering production of gonadotrophin (Gardner, 1955) Luteoma formation was prevented by leaving one ovary intact or by the injection of either oestrogen or androgen Cystic ovaries will be produced by parabiosing a normal to a castrated rodent and in the presence of a carcinogen this may induce ovarian tumours (Bielschowsky and Hall, 1951)

General body irradiation with X rays and gamma rays has been used to produce ovarian cysts and tumours in mice, rats guinea pigs and rabbits (Furth and Boon, 1947 Speert 1952)

A variety of gonadotrophins have been used to induce cyst formation in experimental animals When a gonadotrophin has an inherent follicle stimulating quality, ovarian cyst formation is not an uncommon concomitant of hormone overdosage Chorionic gonadotrophin, however, is essentially a luteinizing hormone in the rodent although a follicular growth quality was observed in the dog

The relationship of thyroid activity to ovarian function provides a further possibility for the spontaneous origin of ovarian cysts altered thyroid secretion can affect the hypophysial gonadal axis Clinical cases of untreated myxoedema exhibit ovarian cysts, and rats made hypothyroid for eight months have a higher percentage of cystic ovaries than do euthyroid rats (Janes 1944) Cystic ovary formation associated with hypothyroidism may be the result of altered hypophysial activity Ovarian cysts in the sow were associated with an increased hypophysial content of thyrotrophic hormone and gonadotrophic hormone (Nalbandov, 1952) The hypothyroid rat ovary is considered more responsive to pregnant mare serum gonadotrophin and to chorionic gonadotrophin than the ovary of euthyroid rats (Meites and Chandrashaker 1949) Chorionic gonadotrophin, known to exert a luteinizing action becomes a follicle stimulator when thyroid activity is subnormal

for the entire experimental period whereas chorionic gonado trophin administration was begun only after a ten day prefeeding period Hypothyroid and euthyroid rats were *autopsied after 5, 10 15 and 20 gonadotrophin injections and* compared with their respective controls

Ovaries of euthyroid animals receiving gonadotrophin were increased fivefold after 5 injections Maximum relative size

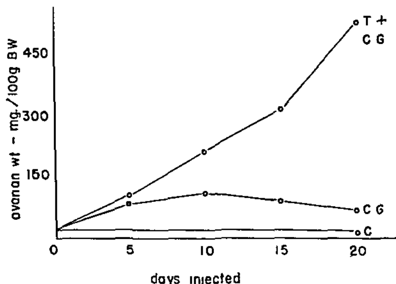


FIG 1 The influence of chorionic gonadotrophin (C G) and thiouracil (T) on ovarian weight in immature rats

was reached after 10 injections followed by a continuous decrease after 15 and 20 injections Thiouracil alone did not influence ovarian weight and chorionic gonadotrophin in hypothyroid rats increased ovarian weight fivefold after 5 injections as in euthyroid rats However continued treat ment resulted in continued ovarian weight increases so that the mean increase after 20 gonadotrophin injections was 2 780 per cent over the weight of the controls and more than 2 000 per cent above that of euthyroid gonadotrophin treated animals (Fig 1)

Little investigation has been applied to the biochemistry of the cystic ovary The data obtained have been taken *largely from post operative or fresh autopsy material and* express a situation at only one point The lipid content of ovarian dermoid cysts has been investigated as well as the physical properties of the lipid Carbohydrates and muco proteins have been identified in cyst fluid (Maki 1952) as well as some polysaccharides with blood group specificity (Grubb and Morgan 1949) Reducing substances were found to be low in proliferating cysts (Schalyt, 1930) while nitrogen protein and potassium were found to be high and chloride low in cyst fluids from active epithelia as compared to cysts with less active epithelia (Watts and Adair, 1944*b*) Perloff and co workers (1955) compared the protein patterns of serum and cyst fluid and found globulin to be lower in the cyst than in serum

With a view towards gaining further information on cyst aetiology, it was deemed of value (1) to devise a method for ovarian cyst formation in the rat and (2) to study the bio chemical changes associated with follicular cyst evolvement

The combination of thiouracil (0 5 per cent) in a 20 per cent casein diet and small doses of chorionic gonadotrophin (10 i u daily for 20 days) following a ten day prefeeding period has repeatedly proved successful for induction of ovarian follicular cysts in rats Neither of these agents is capable in our experience, of inducing cysts by itself An important feature of this method is the use of chorionic gonadotrophin in that individual ovaries weighing 800–1 000 mg are not uncommon Furthermore ovarian cysts once induced do not spontaneously regress

Ovarian weight and histology in cyst induction

Thirty day old female rats were fed a 20 per cent casein diet with or without 0 5 per cent thiouracil and were or were not injected with chorionic gonadotrophin Uniform food intake was maintained by paired feeding Thiouracil was fed

levels in the ovary changed despite administration of gonadotrophin unless the animal was hypothyroid (Table I) In hypothyroidism gonadotrophin stimulation significantly decreased free cholesterol from 0 15 per cent or more to levels of 0 12 per cent or less Thus the physiological circumstances which favoured ovarian cyst induction were associated with a characteristic decrease in free cholesterol Ovaries which were more than 300 mg in weight and exhibited more prominent follicular cysts experienced the more marked free cholesterol changes Cyst fluid contained 18 mg per cent cholesterol virtually all of which was free The serum cholesterol levels were 40 to 60 mg per cent and one third was esterified

Ovarian nucleic acids in cyst induction

Nucleic acids bear a close relationship to cellular metabolism and particularly to protein synthesis In view of the increase in ovarian weight associated with ovarian cyst formation an appraisal of pentose and deoxypentose nucleic acids was made to characterize the ovarian growth in normal and subnormal metabolic states (Nocenti and Leathem 1956)

Chorionic gonadotrophin treatment to normal animals *ad libitum* or pair fed increased ovarian weight and this was accompanied by a proportional rise in total pentose nucleic acid (PNA) phosphorus for PNA P concentration was not altered as compared to control groups at this 20 injection period In contrast the hypothyroid state augmented the chorionic stimulation of PNA synthesis 100 per cent over the injected normal animals but the PNA concentration was lowered by 86 per cent rather than being maintained as in the injected euthyroid animals Chorionic gonadotrophin administered to normal rats caused an increase (47 per cent) in total deoxyribonucleic acid (DNA) phosphorus while the concentration was approximately halved this halving was apparently due to the high cytoplasmic nuclear ratio of the lutein cells (Table II) The total DNA P of the hypothyroid rats receiving chorionic hormone was doubled as compared to

The ovarian weight increase in the hypothyroid rat in response to gonadotrophin was due to increased lutein tissue and to large fluid filled follicles The follicular cysts were lined by simple squamous epithelium in the absence of a granulosa and lutein tissue and were devoid of lipid and alkaline phosphatase

Ovarian cholesterol in cyst induction

Cholesterol is considered to be a precursor for ovarian steroid hormones and ester cholesterol concentration of the gonad varies inversely with hormone synthesis and secretion (Claesson and Hillarp 1946 Levin and Jailer 1948) Unaware of studies concerned with cholesterol content of cystic ovaries this measure was used in an effort to determine whether or not a biochemical characterization of the cystic condition was possible by this measure (Steinetz 1954)

Thiouracil feeding for 30 days did not influence ovarian cholesterol and the ratio of free to total cholesterol was quite constant However the administration of gonadotrophin quickly reduced ester cholesterol in both euthyroid and hypothyroid rats and kept this ovarian component at subnormal levels for the 20 days of chorionic gonadotrophin administration It became apparent too, that free cholesterol

Table I

INFLUENCE OF 20 INJECTIONS OF CHORIONIC GONADOTROPHIN ON OVARIAN WEIGHT AND CHOLESTEROL IN RATS FED A 20% CASEIN DIET WITH AND WITHOUT THIOURACIL

No rats	*Treatment*	*Ovarian wt mg /100 g*	*Cholesterol %* *Total*	*Free*	*Free Total ratio*
8	None	27 [illegible]	1 56	0 18	0 14
8	Thiouracil	21 4	2 29	0 19	0 16
12	Chorionic gonadotrophin	90 8	0 41	0 18	0 46
8[illegible]	Thiouracil + chorionic gonadotrophin	339 9	0 20	0 12	0 63

Cholesterol expressed as percentage of wet organ weight

levels in the ovary changed despite administration of gonado trophin unless the animal was hypothyroid (Table I) In hypothyroidism gonadotrophin stimulation significantly de creased free cholesterol from 0 15 per cent or more to levels of 0 12 per cent or less Thus the physiological circumstances which favoured ovarian cyst induction were associated with a characteristic decrease in free cholesterol Ovaries which were more than 300 mg in weight and exhibited more prominent follicular cysts experienced the more marked free cholesterol changes Cyst fluid contained 18 mg per cent cholesterol virtually all of which was free The serum cholesterol levels were 40 to 60 mg per cent and one third was esterified

Ovarian nucleic acids in cyst induction

Nucleic acids bear a close relationship to cellular metabolism and particularly to protein synthesis In view of the increase in ovarian weight associated with ovarian cyst formation an appraisal of pentose and deoxypentose nucleic acids was made to characterize the ovarian growth in normal and sub normal metabolic states (Nocenti and Leathem 1956)

Chorionic gonadotrophin treatment to normal animals *ad libitum* or pair fed increased ovarian weight and this was accompanied by a proportional rise in total pentose nucleic acid (PNA) phosphorus for PNA P concentration was not altered as compared to control groups at this 20 injection period In contrast the hypothyroid state augmented the chorionic stimulation of PNA synthesis 100 per cent over the injected normal animals but the PNA concentration was lowered by 86 per cent rather than being maintained as in the injected euthyroid animals Chorionic gonadotrophin ad ministered to normal rats caused an increase (47 per cent) in total deoxyribonucleic acid (DNA) phosphorus while the con centration was approximately halved this halving was apparently due to the high cytoplasmic nuclear ratio of the lutein cells (Table II) The total DNA P of the hypothyroid rats receiving chorionic hormone was doubled as compared to

The ovarian weight increase in the hypothyroid rat in response to gonadotrophin was due to increased lutein tissue and to large fluid filled follicles The follicular cysts were lined by simple squamous epithelium in the absence of a granulosa and lutein tissue and were devoid of lipid and alkaline phosphatase

Ovarian cholesterol in cyst induction

Cholesterol is considered to be a precursor for ovarian steroid hormones and ester cholesterol concentration of the gonad varies inversely with hormone synthesis and secretion (Claesson and Hillarp 1946 Levin and Jailer 1948) Unaware of studies concerned with cholesterol content of cystic ovaries this measure was used in an effort to determine whether or not a biochemical characterization of the cystic condition was possible by this measure (Steinetz 1954)

Thiouracil feeding for 30 days did not influence ovarian cholesterol and the ratio of free to total cholesterol was quite constant However the administration of gonadotrophin quickly reduced ester cholesterol in both euthyroid and hypothyroid rats and kept this ovarian component at subnormal levels for the 20 days of chorionic gonadotrophin administration It became apparent too, that free cholesterol

Table I

INFLUENCE OF 20 INJECTIONS OF CHORIONIC GONADOTROPHIN ON OVARIAN WEIGHT AND CHOLESTEROL IN RATS FED A 20% CASEIN DIET WITH AND WITHOUT THIOURACIL

No rats	Treatment	Ovarian wt mg /100 g	Cholesterol % Total	Cholesterol % Free	Free Total ratio
8	None	27 5	1 56	0 18	0 14
8	Thiouracil	21 4	2 29	0 19	0 16
12	Chorionic gonadotrophin	90 8	0 41	0 18	0 46
3ɔ	Thiouracil + chorionic gonadotrophin	339 9	0 20	0 12	0 68

Cholesterol expressed as per ntage of wet organ weight

ovaries was in the inorganic fraction After 20 days of gonadotrophin administration the total ^{32}P uptake of cystic ovaries had decreased and was now only 50 per cent of the uptake observed with a less extensive treatment Furthermore there was no measurable organically bound phosphorus Euthyroid rats receiving the gonadotrophin exhibited an uptake that was less after 20 days of treatment than after 10 but the reduction was less marked (30 per cent) and organically bound isotope was found (Table III)

Table III

^{32}P UPTAKE OF OVARIES FROM HYPOTHYROID AND NORMAL RATS TREATED WITH CHORIONIC GONADOTROPHIN FOR 10 AND 20 DAYS

No rats	*Treatment*	*Ovary A mg /100 g*	*Ovary B mg /100 g*	*Total ^{32}P**	*Acid soluble*
8	Thiouracil+chorionic gonadotrophin × 10	139	132	15 7	13 2
8	Chorionic gonadotrophin × 10	74	57	8 1	5 8
11	Thiouracil+chorionic gonadotrophin × 20	217	145	8 0	7 0
10	Chorionic gonadotrophin × 20	41	45	5 6	3 7

CPM/mg 100 g /inj dose × 10⁻

The data suggest that the ^{32}P is signifying energy expenditure and may be related to a synthetic process At the time when ovarian growth rate was most rapid (10 injection period) the ^{32}P uptake was highest When cysts were well established and the size increments were less pronounced (20 injection period) ^{32}P uptake had decreased to half No such decrease occurred in euthyroid rats although acid soluble radiophosphorus uptake was significantly lower

Lactic acid in cyst induction

Carbohydrate metabolism is important to the normal functioning of ovarian tissue Gonadotrophic hormones stimulate the ovary to increase tissue growth and steroid

Table II

INFLUENCE OF 20 INJECTIONS OF CHORIONIC GONADOTROPHIN ON OVARIAN WEIGHT AND NUCLEIC ACID PHOSPHORUS OF RATS FED A 20% CASEIN DIET WITH AND WITHOUT THIOURACIL

No rats	*Treatment*	*Ovarian wt mg /100 g*	*PNA P Total*	*PNA P Conc ‡*	*DNA P Total*	*DNA P Conc*
7*	None	12 6	0 003	0 010	0 025	0 166
6*	Thiouracil	18 7	0 008	0 037	0 031	0 156
7*	Chorionic gonadotrophin	52 3	0 018	0 032	0 042	0 073
9	Thiouracil + chorionic gonadotrophin†	566 3	0 040	0 005	0 096	0 0[illegible]2
11	Chorionic gonado trophin	47 4	0 020	0 031	0 045	0 079
10	None	19 7	0 010	0 033	0 031	0 1[illegible]4

Pair fed
† Chorionic gonadotrophin injected day 10–30
Conc = mg /100 mg tissue

the normal injected rats while the DNA P concentration was 70 per cent lower than that in ovaries from normal injected rats Thus cystic ovaries exhibit an increased PNA and DNA synthesis The PNA P and DNA P concentrations were decreased by the diluting effect of cyst fluid

The uptake of ^{32}P in cyst induction

The maximum uptake of ^{32}P by rabbit and mouse ovaries occurred at times when steroid synthesis was highest (Odeblad 1951 Albert and Johnson, 1952) Furthermore active grow ing tissues were found to accumulate ^{32}P while atrophic tissues did not (Gennaro 1954) Ovaries of normal and hypothyroid rats injected with chorionic gonadotrophin for 10 and 20 days were studied four hours following the sub cutaneous injection of 60 μc of radiophosphorus One ovary (A) was digested for total ^{32}P and the other ovary (B) was extracted for acid soluble ^{32}P After 10 injections of chorionic gonadotrophin the ovarian four hour uptake of total ^{32}P in hypothyroid rats was nearly twice that of ovaries of euthyroid rats Eighty four per cent of the ^{32}P uptake of the cystic

Thiouracil induced hypothyroidism influenced ovarian lactic acid significantly Total ovarian lactic acid was 16 1 µg greater in hypothyroid rats and the concentration increased 1 1 µg ($P < 0$ 01) in comparison with pair fed euthyroid rats After 20 daily injections of chorionic gonadotrophin to hypothyroid animals total ovarian lactic acid increased from 79 µg in control rats to 158 µg in gonadotrophin treated animals Lactic acid concentration decreased from 4 3 µg to 0 4 µg per mg of wet tissue in hypothyroid rats in response to chorionic hormone Hypothyroid rat ovaries respond to gonadotrophin with a greater increase in total lactic acid and a more profound decrease in lactic acid concentration than euthyroid rat ovaries The observed decrease in lactic acid concentration indicated that hypothyroidism alters carbohydrate metabolism in the ovary Further it would appear likely that lactic acid is oxidized in the hypothyroid rat ovary to a greater degree than that observed in the euthyroid rat ovary and thus serves as an energy source for enhanced protein synthesis and steroid elaboration In addition it has been determined that oxygen uptake and succinate oxidation of the rat ovary are not influenced by hypothyroidism meaning probably that aerobic oxidation has not been impaired (Barker and Schwartz 1953 Barker 1955) The increased total lactic acid seems related to increased lactic acid production or the failure of lactate conversion to pyruvate Significantly more lactic acid was present in the ovarian residue than in the cyst fluid but lactic acid concentrations were identical for each

Rat cystic fluid hormones

The immature mouse uterine epithelium contains sudanophilic lipid which will disappear in 72 hours following the administration of 0 2 µg of oestradiol The test is reasonably specific as androgen progesterone and cortisone in dosages of 100 fold larger than oestradiol had no effect Several tests with cyst fluid in amounts as small as 0 1 ml gave a positive

synthesis, and carbohydrate metabolism supplies in part at least, the energy source for the synthetic processes Lactic acid was chosen as one measure of carbohydrate metabolism and in this regard, Lutwak Mann (1954) found lactic acid in cow follicular fluid and suggested that lactic acid may reflect local glycolytic activity of follicular cells Furthermore *in vitro* studies of normal and tumorous tissue indicate that lactic acid is produced in an anaerobic system, but is reduced three to six times by the addition of oxygen suggesting the capacity of normal and abnormal tissues for oxidizing lactic acid (Weinhouse, 1951) A fundamental difference between normal tissue and neoplasms is the characteristic ability of tumours to produce larger amounts of lactic acid suggesting an increased anaerobic glycolysis rather than a decreased aerobic carbohydrate metabolism

Lactic acid is present in measurable amounts in the rat ovary and total lactic acid increases with the growth of this organ (Greslin and Leathem, 1957) Ovarian stimulation induced with chorionic gonadotrophin in euthyroid rats increased total ovarian lactic acid but reduced lactic acid concentration to 1 μg from the 3 2 μg concentration of untreated rats (Table IV)

Table IV

INFLUENCE OF CHORIONIC GONADOTROPHIN ON OVARIAN LACTIC ACID IN NORMAL AND HYPOTHYROID RATS

No rats	Treatment	Ovarian wt mg	Lactic acid Total (μg)	Lactic acid (μg/mg)
10	Control*	12 [illegible]	29 [illegible]	2 3
10	20% Casein	19 6	62 9	3 2
11	Thiouracil	18 3	79 0	4 3
10	Chorionic gonadotrophin	110 0	112 1	1 0
18	Thiouracil+chorionic gonadotrophin	375 9	157 6	0 4

Initial 30-day-old control
Thiouracil fed f r 30 days
Chorionic gonadotrophin injected for 0 days

Thiouracil induced hypothyroidism influenced ovarian lactic acid significantly Total ovarian lactic acid was 16 1 µg greater in hypothyroid rats and the concentration increased 1 1 µg ($P<0$ 01) in comparison with pair fed euthyroid rats After 20 daily injections of chorionic gonadotrophin to hypothyroid animals total ovarian lactic acid increased from 79 µg in control rats to 158 µg in gonadotrophin treated animals Lactic acid concentration decreased from 4 3 µg to 0 4 µg per mg of wet tissue in hypothyroid rats in response to chorionic hormone Hypothyroid rat ovaries respond to gonadotrophin with a greater increase in total lactic acid and a more profound decrease in lactic acid concentration than euthyroid rat ovaries The observed decrease in lactic acid concentration indicated that hypothyroidism alters carbohydrate metabolism in the ovary Further it would appear likely that lactic acid is oxidized in the hypothyroid rat ovary to a greater degree than that observed in the euthyroid rat ovary and thus serves as an energy source for enhanced protein synthesis and steroid elaboration In addition it has been determined that oxygen uptake and succinate oxidation of the rat ovary are not influenced by hypothyroidism meaning probably that aerobic oxidation has not been impaired (Barker and Schwartz 1953 Barker 1955) The increased total lactic acid seems related to increased lactic acid production or the failure of lactate conversion to pyruvate Significantly more lactic acid was present in the ovarian residue than in the cyst fluid but lactic acid concentrations were identical for each

Rat cystic fluid hormones

The immature mouse uterine epithelium contains sudanophilic lipid which will disappear in 72 hours following the administration of 0 2 µg of oestradiol The test is reasonably specific as androgen progesterone and cortisone in dosages of 100 fold larger than oestradiol had no effect Several tests with cyst fluid in amounts as small as 0 1 ml gave a positive

reaction for oestrogen In a single testing using the Hooker Forbes test for progesterone a positive response was obtained

Ovarian cyst regression patterns

If the experimental cysts are to be used in the search and study of aetiological and therapeutic factors, then characteristics of the regression pattern must be determined Spontaneous ovarian cysts which become clinical and veterinarian problems may or may not regress spontaneously Since our experimental design involves the administration of chorionic gonadotrophin the post injection phase can be examined for cyst regression Examination of both ovaries a day after the last injection of chorionic hormone and the removal of one ovary has shown that a tendency for spontaneous regression is greater if the initial stimulation did not increase the weight of one ovary above 200 mg Despite correction of the subnormal thyroid state, cystic ovaries have persisted for 15 months after cessation of gonadotrophin and ovaries weighing five to seven grams have been obtained

Groups of immature rats in which cyst induction was attempted were held for six months on thiouracil Seven of 12 rats became pregnant on mating but only three carried young to weaning The other litters were destroyed by the mother in a few days The ovaries of these animals were cystic In two animals, one ovary was normal whereas the opposite ovary weighed 7 2 g and 5 6 g Seven adult rats were treated to induce ovarian cysts then returned to a normal diet for one month Mating attempts at this time produced only three pregnancies

Although ovarian weight and gross appearance of follicular cysts have provided information on regression patterns it was considered possible that ovarian biochemical composition may or may not be returning to normal and these analyses would then provide a more definitive clue

Immature female rats were fed the 20 per cent casein diet containing 0 5 per cent thiouracil After 10 days the rats

were injected daily for 20–30 days with 10 i u of chorionic gonadotrophin After the last injection, the animals were unilaterally ovariectomized by removing the smaller ovary This ovary was weighed and designated ovary A for determination of cholesterol During the post injection period the rats were fed a diet from which the thiouracil had been removed and 0 1 per cent thyroid powder added After 20 days the rats were sacrificed, ovary B obtained for weight and cholesterol and the thyroid and adrenal weights recorded as a measure of the return of these organ weights to normal The data are presented in Table V by grouping of ovarian

Table V

INFLUENCE OF 0 1 % THYROID ON OVARIAN WEIGHT AND CHOLESTEROL DURING 20 DAYS OF CYST REGRESSION

No rats	Ovary A wt mg	Cholesterol % Total	Cholesterol % Free	Ovary B wt mg	Cholesterol % Total	Cholesterol % Free
12	147	0 27	0 16	97	0 43	0 15
2	233	0 22	0 15	155	0 27	0 12
5	396	0 11	0 07	537	0 15	0 07
4	739	0 09	0 04	316	0 19	0 08

cysts in different weight ranges Total ovarian cholesterol was reduced from the 0 80 per cent levels of controls to 0 27 per cent or less by the experimental development of ovarian cysts Depression of total cholesterol was greatest in the largest ovaries Free cholesterol was subnormal only in the ovaries weighing 300 mg or more Following 20 days of thyroid feeding thyroid and adrenal weights returned to normal and in some instances ovarian weight did return towards normal Total ovarian cholesterol increased somewhat in each group but the free cholesterol which is suggestively more characteristic of cystic ovaries was not corrected towards normal and in most instances cysts were not abolished Dosage and types of thyroid preparations may provide more encouraging results Nevertheless the lack of spontaneous regression and correction of ovarian cholesterol provide a

more firm basis upon which to study the factors which may aid the return of ovarian composition and function to normal

Acknowledgements

Grateful acknowledgement is made to Doctors Bernard G Steinetz Mero R Nocenti and Joseph R Greslin whose thesis researches make this report possible

Chorionic gonadotrophin Follutein was generously supplied by E R Squibb & Sons New Brunswick New Jersey

REFERENCES

ALBERT S and JOHNSTON R M (1952) *Cancer Res* 12 584
BARKER S B (1955) *Endocrinology* 57 414
BARKER S B and SCHWARTZ H B (1953) *Proc Soc exp Biol N Y* 83 500
BIELSCHOWSKY F and HALL W H (1951) *Brit J Cancer* 5 331
CASIDA L E CASIDA B R and CHAPMAN A B (1951) *J Dairy Sci* 34 1200
CLAESSON L and HILLARP N (1946) *Acta physiol scand* 13 115
CRELIN E S and WOLSTENHOLME J T (1951) *Cancer Res* 11 212
ENGEL L L DORFMAN R I and ABARBANEL A R (1953) *J clin Endocrin Metab* 13 903
FISHER R H and RILEY C L (1952) *J clin Endocrin Metab* 12 890
FURTH J and BOON M C (1947) *Cancer Res* 7 241
GARDNER W U (1955) *Cancer Res* 15 109
GARM O (1949) *Cornell Vet* 39 39
GENNARO J F Jr (1954) *Anat Rec* 118 301
GREEN J A (1956) *Cancer Res* 16 417
GREEN J A (1957) *Cancer Res* 17 86
GRESLIN J and LEATHEM J H (1957) *Fed Proc* 16 51
GRUBB R and MORGAN W T J (1949) *Brit J exp Path* 30 198
JANES R G (1944) *Anat Rec* 90 93
KLEINFELTER H F and SEEGER JONES G E (1954) *J clin Endocrin Metab* 14 1247
KULLANDER S (1956) *Acta endocr Copenhagen* 23 131
LEVIN L and JAILER J W (1948) *Endocrinology* 43 154
LUTWAK MANN C (1954) *J agric Sci* 44 477
MAKI M (1952) *Tohoku J exp Med* 55 331
MEITES J and CHANDRASHAKER B (1949) *Endocrinology* 4 368
NALBANDOV A V (1952) *Fertility and Sterility* 3 100
NOCENTI M R and LEATHEM J H (1956) *Proc Pa Acad Sci* 30 24
ODEBLAD E (1951) *Scand J clin Lab Invest* 3 151
PARKES A S (1950) *Recent Progr Hormone Res* 5 101

PERLOFF W H SCHULTZ J FARRIS E J and BALIN H (1955) *Fertility and Sterility* 6 11
SCHALYT L G (1930) *Arch Gynaek* 139 614
SPEERT H J (1949) *J clin Endocrin* 9 630
SPEERT H J (1952) *Cancer N Y* 5 478
STARKEY W F and LEATHEM J H (1943) *Anat Rec* 86 401
STEINETZ B G (1954) Ph D Thesis Rutgers University
WATTS R M and ADAIR F L (1944a) *Amer J Obstet Gynec* 47 593
WATTS R M and ADAIR F L (1944b) *Amer J Obstet Gynec* 48 1
WEINHOUSE S (1951) *Cancer Res* 11 585
WEST R H (1955) *Quart Bull Northw Univ med Sch* 29 256
ZONDEK B and SKLOW J (1942) *J Endocrin* 3 1

DISCUSSION

Gardner Were the data compiled from the figures for wet weights or dry weights?

Leathem This was computed in mg per wet weight If you are concerned about fluid concentrations we have also done dry weights and this does not make any difference

We have been completely unsuccessful with thyroid alone correcting the thyroid state making them hyperthyroid for periods of six months or one month has not influenced the cystic ovaries at all We have just tried this one pattern and of course it is dangerous to make any conjectures upon it—feeding thyroid for the first ten days then administering growth hormone for the following ten days is the first evidence that we have of bringing ovarian cholesterol back to what we would expect of the normals

Huseby The growth hormone helps to restore the cholesterol but does it affect the histology?

Leathem No if we examine these ovaries morphologically then we still have cysts present On the other hand I think that if we can correct the biochemical aspects of the ovary we have a better chance of the regression of cysts If we have just a morphological picture we do not really know whether we are getting the ovarian tissue back to normal

Luft Prof Leathem isn t this ovarian picture very similar to the one we see in Cushing s syndrome and virilism?

Leathem I have not seen this Perhaps the pathologists could say something about it

Symington I have not seen cystic ovaries in Cushing s syndrome but I think Dr Furth will agree that this question of cystic ovaries is quite a problem Some of the ovaries we have seen have cysts with a flattened lining and such cysts do not seem to be functioning Other cysts are lined by granulosa like cells and I have always wondered what exactly they were doing I do not know of any work that has been done on such cystic ovaries and that is why I was interested in this presentation

Luft We have seen this in quite a number of cases not only the flattened cells but also luteinization of the walls and of the interstitial cells Even in virilization without signs of Cushing s syndrome some cases have luteinized cysts in the ovaries

Pincus Have you studied the effect of hypophysectomy on the life and/or death of these cystic ovaries?

Leathem No the only thing we have done with the hypophysectomy is to discover that you cannot develop cystic ovaries with this pattern in the hypophysectomized rat We have been unable to develop them when we give growth hormone TSH FSH thiouracil and chorionic gonadotrophin all at one time

Gardner You mentioned that from the earliest experiments the same changes occurred in thyroidectomized animals Is that consistent? Do you observe anything specific with thiouracil other than the thyroid deficiency?

Leathem We got nothing specific with thiouracil I think that the basic difference here is that we get a hypothyroid state because we have created it by thyroidectomy by thiocyanate by sulphaguanidine and by thiouracil and in each instance we get the same morphological pattern and the same ovarian cholesterol changes Therefore we have gone on using basically thiouracil *Of course it did occur to us initially* that thiouracil as a drug might have some unique actions in its own right It certainly acts upon the liver in a manner different to thyroidectomy I am still curious though about why we can correct the thyroid hypertrophy induced by thiouracil and not prevent cyst formation For the latter we must go to higher doses Barker has suggested that hyperthyroidism does not change the metabolic state of the ovary and if this is correct then we should not be using thyroid hormone

Tata Did you measure any blood cholesterol and compare it with the free and total cholesterol in the ovaries?

Leathem Yes the cyst fluid has almost no free cholesterol—in five instances out of ten it was not detectable at all Total cholesterol was very much less in both the ovary and in cyst fluid than in the circulating serum

Tata When you change the thyroid function is there a very marked change in blood cholesterol levels and are these thyroid changes parallel to the blood cholesterol level changes?

Leathem Very frequently they were not and I am fairly convinced that they are not connected

Crooke The production of follicles by luteinizing hormone puzzles me Have you used pure luteinizing hormones?

Leathem We used materials supplied by the Armour Company

Crooke This is still human chorionic gonadotrophin?

Leathem No it was interstitial cell or luteinizing hormone from the pituitary gland I cannot answer for the purity except from our own designations and our own efforts in hypophysectomized rats to see how it might work The dosages which we used which were relatively small seemed to be fairly uncontaminated The material which we settled on for most of these experiments was human chorionic gonadotrophin of a

potency of a little under 3 000 units per mg and at the dosages which we were using 10 i u per day for 20 days at the most it seemed to be a fairly uniform material

Crooke That still may contain a significant amount of follicle stimulating hormone?

Leathem It may very well The fact that we have given this dosage and ten times this dosage to hypophysectomized rats and have not been able to budge the ovaries at all I think shows that the material is at least not highly contaminated with FSH We were suspicious of this and had to resort to doing a trial with hypophysectomized rats to suggest that these dosages were not all the result of FSH contamination I do not believe that they are I do not deny the fact though that when we change the metabolic stature of the animal and maybe even that of the ovaries specifically these cells might be more sensitive to one gonadotrophin than to the other This would be an interesting pattern in itself even if the preparation were contaminated because it would mean that something spontaneous could similarly occur under the influence of its own pituitary gland

Pincus You have not expressly tested FSH versus LH in the thiouracil treated animal?

Leathem No

Muhlbock Have you ever transferred the ovaries to another place?

Leathem No I am sorry to say we have not done that Dr Muhlbock This is something I keep thinking we ought to do and then we go off into some other aspect of the biochemistry

Muhlbock I had an idea that if you transplanted subcutaneously it would very much favour the development of these cysts

Gardner Can you get the same responses in mice? Is this consistent with different strains of rats or is the response strain limited?

Leathem We have tried this on C3H and in Swiss mice and they do not develop cysts In fact enhanced ovarian stimulation can be gained in mice by giving a little bit of thyroid rather than making them hypothyroid

In answer to the second question we have done this experiment in Long Evans Wistar Sprague Dawleys and some bizarre strain which is sold to us by a breeder All of them are fairly consistent

GONADOTROPHINS IN CASES OF HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA OF THE UTERUS

CHRISTIAN HAMBURGER

Hormone Department Statens Seruminstitut Copenhagen

QUANTITATIVE and semi quantitative assays of the urinary excretion of chorionic gonadotrophin in 150 cases of hydatidiform mole and chorionepithelioma (ch ep) of the uterus were carried out by Hamburger and Terkildsen (1948) As the complete material was published in the Danish language only, a brief summary of the investigation is given here

In 29 cases the urines were examined before the mole was removed Eighty six per cent of the patients excreted more than 300 000 i u of chorionic gonadotrophin per litre or per day, the maximum excretion being 20 million i u per litre Among 135 specimens of urine from normal pregnancies, 6 per cent contained above 300 000 i u In one of these cases an excretion of 900 000 i u led to evacuation of the uterus with removal of a normal foetus! Although the output was on the average much higher in the cases of moles the values overlap to such an extent that the quantitative determinations of the gonadotrophin excretion have little if any value for the diagnosis of a molar pregnancy

On the other hand after the expulsion or removal of a hydatidiform mole regular control of the gonadotrophin excretion is strongly advised In this connexion it is important to recollect that it is much longer before the gonadotrophin disappears from the urine after a molar pregnancy than after a normal pregnancy In our 96 uncomplicated cases of hydatidiform mole the excretion persisted for 1 2 and 3 months in 47 23 and 10 per cent respectively while none of the patients

examined excreted chorionic gonadotrophin half a year after the removal of the mole

In 39 of the cases various complications occurred viz, 18 cases of ch ep 7 cases of histologically doubtful ch ep 6 cases of remnants of molar tissue 2 cases with a new and unrecognized pregnancy and 6 uncertain cases

These cases were characterized by one or more of the following features (1) increase of the gonadotrophin excretion (2) protracted excretion (i e more than half a year) and (3) a content of more than 30 000 i u at least one month after removal of the mole The quantitative gonadotrophin analyses give however no information as to the nature of the complication—they indicate only if something is going wrong

A survey of the 18 cases of ch ep is given in Table I Twelve of the patients recovered after ablation of the uterus or curettage In four of the cases the observation period was rather short but it is a well known fact that in the fatal cases death usually occurs within two years Six patients i e one third of the ch ep cases died four of them with metastases

The relatively good prognosis of even histologically malignant ch ep in women has been stressed by Hertig and Sheldon (1947) among others In this respect the ch ep of the uterus differs markedly from ch ep of the testis and other testicular tumours producing chorionic gonadotrophin It is extremely rare that a patient with a testis tumour is alive one or two years after the development of tissue elements producing chorionic gonadotrophin (cf Hamburger 1957)

In his interesting thesis for the doctorate den Hartog (1933) pointed out that only *male* germ cells but not the ripe oöcyte possess the ability to form chorionic epithelium Therefore ch ep in the female always originates from a fertilized egg cell or in cases of the rare ovarian ch ep from testicular remnants in the ovary In both sexes ch ep is a male ' tumour

Hertig and Sheldon (1947) recall the fact that a tumour derived from trophoblast differs from any other tumour in this respect its benign prototype is normally invasive opens

GONADOTROPHINS IN CASES OF HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA OF THE UTERUS

Christian Hamburger

Hormone Department Statens Seruminstitut Copenhagen

Quantitative and semi quantitative assays of the urinary excretion of chorionic gonadotrophin in 150 cases of hydatidiform mole and chorionepithelioma (ch ep) of the uterus were carried out by Hamburger and Terkildsen (1948) As the complete material was published in the Danish language only, a brief summary of the investigation is given here

In 29 cases the urines were examined before the mole was removed Eighty six per cent of the patients excreted more than 300 000 i u of chorionic gonadotrophin per litre or per day the maximum excretion being 20 million i u per litre Among 135 specimens of urine from normal pregnancies, 6 per cent contained above 300 000 i u In one of these cases an excretion of 900 000 i u led to evacuation of the uterus with removal of a normal foetus[1] Although the output was on the average much higher in the cases of moles the values overlap to such an extent that the quantitative determinations of the gonadotrophin excretion have little if any value for the diagnosis of a molar pregnancy

On the other hand after the expulsion or removal of a hydatidiform mole regular control of the gonadotrophin excretion is strongly advised In this connexion it is important to recollect that it is much longer before the gonadotrophin disappears from the urine after a molar pregnancy than after a normal pregnancy In our 96 uncomplicated cases of hydatidiform mole the excretion persisted for 1 2 and 3 months in 47 23 and 10 per cent respectively while none of the patients

examined excreted chorionic gonadotrophin half a year after the removal of the mole

In 39 of the cases various complications occurred viz, 18 cases of ch ep 7 cases of histologically doubtful ch ep 6 cases of remnants of molar tissue 2 cases with a new and unrecognized pregnancy, and 6 uncertain cases

These cases were characterized by one or more of the following features (1) increase of the gonadotrophin excretion (2) protracted excretion (i e more than half a year) and (3) a content of more than 30 000 i u at least one month after removal of the mole The quantitative gonadotrophin analyses give however no information as to the nature of the complication—they indicate only if something is going wrong

A survey of the 18 cases of ch ep is given in Table I Twelve of the patients recovered after ablation of the uterus or curettage In four of the cases the observation period was rather short but it is a well known fact that in the fatal cases death usually occurs within two years Six patients i e one third of the ch ep cases died four of them with metastases

The relatively good prognosis of even histologically malignant ch ep in women has been stressed by Hertig and Sheldon (1947) among others In this respect the ch ep of the uterus differs markedly from ch ep of the testis and other testicular tumours producing chorionic gonadotrophin It is extremely rare that a patient with a testis tumour is alive one or two years after the development of tissue elements producing chorionic gonadotrophin (cf Hamburger 1957)

In his interesting thesis for the doctorate den Hartog (1933) pointed out that only *male* germ cells but not the ripe oöcyte, possess the ability to form chorionic epithelium Therefore ch ep in the female always originates from a fertilized egg cell or in cases of the rare ovarian ch ep from testicular remnants in the ovary In both sexes ch ep is a 'male' tumour

Hertig and Sheldon (1947) recall the fact that a tumour derived from trophoblast differs from any other tumour in this respect its benign prototype is normally invasive opens

GONADOTROPHINS IN CASES OF HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA OF THE UTERUS

CHRISTIAN HAMBURGER

Hormone Department Statens Seruminstitut Copenhagen

QUANTITATIVE and semi quantitative assays of the urinary excretion of chorionic gonadotrophin in 150 cases of hydatidiform mole and chorionepithelioma (ch ep) of the uterus were carried out by Hamburger and Terkildsen (1948) As the complete material was published in the Danish language only, a brief summary of the investigation is given here

In 29 cases the urines were examined before the mole was removed Eighty six per cent of the patients excreted more than 300 000 i u of chorionic gonadotrophin per litre or per day the maximum excretion being 20 million i u per litre Among 135 specimens of urine from normal pregnancies, 6 per cent contained above 300 000 i u In one of these cases an excretion of 900 000 i u led to evacuation of the uterus with removal of a normal foetus! Although the output was on the average much higher in the cases of moles the values overlap to such an extent that the quantitative determinations of the gonadotrophin excretion have little if any value for the diagnosis of a molar pregnancy

On the other hand after the expulsion or removal of a hydatidiform mole regular control of the gonadotrophin excretion is strongly advised In this connexion it is important to recollect that it is much longer before the gonadotrophin disappears from the urine after a molar pregnancy than after a normal pregnancy In our 96 uncomplicated cases of hydatidiform mole the excretion persisted for 1, 2 and 3 months in 47 23 and 10 per cent respectively while none of the patients

examined excreted chorionic gonadotrophin half a year after the removal of the mole

In 39 of the cases various complications occurred viz 18 cases of ch ep 7 cases of histologically doubtful ch ep 6 cases of remnants of molar tissue, 2 cases with a new and unrecognized pregnancy and 6 uncertain cases

These cases were characterized by one or more of the following features (1) increase of the gonadotrophin excretion (2) protracted excretion (i e more than half a year) and (3) a content of more than 30 000 i u at least one month after removal of the mole The quantitative gonadotrophin analyses give however no information as to the nature of the complication—they indicate only if something is going wrong

A survey of the 18 cases of ch ep is given in Table I Twelve of the patients recovered after ablation of the uterus or curettage In four of the cases the observation period was rather short but it is a well known fact that in the fatal cases death usually occurs within two years Six patients i e one third of the ch ep cases died four of them with metastases

The relatively good prognosis of even histologically malignant ch ep in women has been stressed by Hertig and Sheldon (1947) among others In this respect the ch ep of the uterus differs markedly from ch ep of the testis and other testicular tumours producing chorionic gonadotrophin It is extremely rare that a patient with a testis tumour is alive one or two years after the development of tissue elements producing chorionic gonadotrophin (cf Hamburger 1957)

In his interesting thesis for the doctorate den Hartog (1933) pointed out that only *male* germ cells but not the ripe oocyte possess the ability to form chorionic epithelium Therefore ch ep in the female always originates from a fertilized egg cell or in cases of the rare ovarian ch ep from testicular remnants in the ovary In both sexes ch ep is a 'male tumour

Hertig and Sheldon (1947) recall the fact that a tumour derived from trophoblast differs from any other tumour in this respect its benign prototype is normally invasive opens

Table I

CASES OF CHORIONEPITHELIOMA OF THE UTERUS
TREATMENT AND CLINICAL COURSE

Pt No	Original diagnosis	Interval between evacuation of uterus and treatment	Treatment	Histological diagnosis	Clinical course (period of observation)
2	Hydat mole	4 months	Total hysterectomy	Ch ep malign (atypical)	Recovery 6 yrs
7	Hydat mole	1 5	Supravaginal amputation	Ch ep incip (atypical)	Recovery 5
9	Hydat mole	2	Supravaginal amputation	Ch ep	Recovery 5
8	Hydat mole	1	Supravaginal amputation	Ch ep	Recovery 4
3	Hydat mole	4	Total hysterectomy + salpingo oophorectomy + radiotherapy	Ch ep	Recovery 3
1	Hydat mole	17	Hysterectomy	Ch ep	Recovery 3
1	Hydat mole (tendency to ch ep)	0 5	Total hysterectomy	Ch ep incip	Recovery
5	Hydat mole	1	Total hysterectomy	Ch ep malign	Recovery 2
8	Hydat mole	2 5	Hysterectomy + salpingo oöphorectomy (double)	Ch ep	Recovery ?
4	Hydat mole (Ch ep)	2	Total hysterectomy	Ch ep	Recovery 1
8	Hydat mole	1 5	Curettage + radiotherapy	Ch ep	Recovery 6
4	Placenta normal	2 5	Curettage	Ch ep	Recovery 4
3	Hydat mole	28	Supravaginal amputation	Ch ep	Died 28 months
0	Hydat mole	4 5	Curettage	Pulmonary metastases	Died 11
2	Hydat mole	8	Subtotal hysterectomy	Ch ep Pulmonary metastases	Died 16
0	Hydat mole (Ch ep)	immediately	Vaginal hysterectomy + radiotherapy	Vaginal metastases	Died 20
4	Abortion (endometritis)	9 months	Salpingo oöphorectomy (Lt) + salpingectomy (Rt)	Ch ep malign	Died 10
[illegible]	Abortion (endometritis)	1	Total hysterectomy + *salpingo oöphorectomy* (double) + radiotherapy	Ch ep Pulmonary metastases	Died 8

and permeates blood vessels, and thereby often metastasizes to the lungs This however applies to ch ep in both sexes and does not explain the fundamental difference between the uterine and the testis ch ep It seems as if the female organism possesses means of combating the dangerous growth of trophoblastic tissues of which the male organism is devoid It would be interesting to study whether some enzymic factor might be involved

REFERENCES

DEN HARTOG B J C (1933) Het chorionepithelioma malignum van den man en zijn biologische beteekenis Thesis Amsterdam J H de Bussy

HAMBURGER C (1957) This volume p 200

HAMBURGER C and TERKILDSEN K (1948) *Ugeskr Læg* 110 423

HERTIG A T and SHELDON W H (1947) *Amer J Obstet Gynec* 53 1

[Discussion after this paper was postponed until after the short communication by Prof Boyland —EDS]

Short Communication

EFFECT OF PITUITARY ABLATION ON GONADOTROPHIN EXCRETION IN WOMEN WITH BREAST CANCER

E Boyland

Chester Beatty Research Institute London

My clinical colleagues Mr W P Greening Dr P Rigby Jones and Dr J J Stevenson have been developing the operation of pituitary ablation for treatment of cancer of the breast in women Small radioactive rods of gold (^{198}Au) or yttrium (^{90}Y) are inserted into the hypophysis through a long needle inserted through the nares under radiographic control In order to assess the effectiveness of the procedure some measure of pituitary function is necessary For this reason we have estimated the urinary excretion of human menopausal gonadotrophin (HMG) by the method developed in the Medical Research Council Clinical Endocrinology Research Unit in the University of Edinburgh by Dr J A Loraine to whom we are indebted for help and advice The estimations were carried out by Dr M A M Abul Fadl and Miss B Godsmark in the Endocrinology Laboratory of the Royal Marsden Hospital using the method of Loraine and Brown (1954) with the modification that the increase in weight rather than the weight itself of the mouse uterus was used in the bioassay The effect on the uterine weight was compared with the effect produced by a standard preparation of HMG which in turn was standardized against the preparation HMG100 of which 1 mg is one unit We are indebted to Messrs Organon Laboratories for the HMG preparations which were used as internal standards

The results obtained in 30 cases are tabulated (Table I) according to the clinical response which treatment produced Of the 12 cases in which no improvement in clinical conditions was observed four (nos 6 7, 8 and 11) were still excreting at least 24 units of HMG per day after treatment in these cases the treatment had probably been ineffective in destroying the pituitary cells One of the cases showing no improvement (no 1) was excreting only four units of HMG and one showing good improvement (no 22) was excreting only one unit before treatment and in these cases the method cannot be used as an indication of change in pituitary function In six of the cases which did not improve on treatment (nos 3 4 5 9 10 and 12)

Table I

EFFECTS OF IMPLANTATION OF RADIOACTIVE MATERIAL INTO THE PITUITARY ON THE EXCRETION OF HMG AND THE CLINICAL CONDITION OF WOMEN WITH CANCER OF THE BREAST

No	Age	Material Implanted	HMG excretion (units per 24 hours) Before Implantation	After Implantation	Effect on HMG Excretion	Improvement in clinical condition patient following treatment
1	72	Y	4	5 3	?	
2	56	Y	10	5 3	?	
3	54	Y	12	0	+++	
4	68	Y	19	0	+++	
5	47	Y	23	3	+++	
6	51	Y	24	24	0	None
7	49	Au	40	24	+	
8	43	Y	45	38	0	
9	58	Au	50	1	+++	
10	37	Y	80	10	++	
11	69	Au	100	25	++	
12	69	Y	120	5	+++	
13	58	Y	14	0	+++	
14	65	Au	18	33	0	
15	67	Au	30	30	0	
16	48	Y	48	2	+++	Slight
17	62	Y	96	40	++	
18	40	Y	111	6	+++	
19	55	Au	120	23	+	
20	31	Y	192	32	+	
21	48	Au	48	55	0	Moderate
22	54	Au	1	1	?	
23	49	Y	10	1	+++	
24	76	Y	17	8	++	
25	63	Y	25	0	+++	
26	43	Au	30	5	+++	Good
27	41	Au	35	3	+++	
28	37	Y	35	2	+++	
29	54	Y	46	11	+++	
30	46	Au	60	5	+++	

the gonadotrophin excretion fell by at least 90 per cent the tumours in these cases were presumably independent of pituitary hormones

Of the nine cases in which a good clinical response to the pituitary ablation was observed one (no 22 already mentioned) was excreting only one unit of HMG but all the other eight cases (nos 23–30) showed considerable falls in gonadotrophin excretion

The changes in gonadotrophin excretion among the nine patients showing slight or moderate improvement are less clear-cut than in

Short Communication

EFFECT OF PITUITARY ABLATION ON GONADOTROPHIN EXCRETION IN WOMEN WITH BREAST CANCER

E Boyland

Chester Beatty Research Institute London

My clinical colleagues Mr W P Greening Dr P Rigby Jones and Dr J J Stevenson have been developing the operation of pituitary ablation for treatment of cancer of the breast in women Small radioactive rods of gold (^{198}Au) or yttrium (^{90}Y) are inserted into the hypophysis through a long needle inserted through the nares under radiographic control In order to assess the effectiveness of the procedure some measure of pituitary function is necessary For this reason we have estimated the urinary excretion of human menopausal gonadotrophin (HMG) by the method developed in the Medical Research Council Clinical Endocrinology Research Unit in the University of Edinburgh by Dr J A Loraine to whom we are indebted for help and advice The estimations were carried out by Dr M A M Abul Fadl and Miss B Godsmark in the Endocrinology Laboratory of the Royal Marsden Hospital using the method of Loraine and Brown (1954) with the modification that the increase in weight rather than the weight itself of the mouse uterus was used in the bioassay The effect on the uterine weight was compared with the effect produced by a standard preparation of HMG which in turn was standardized against the preparation HMG100 of which 1 mg is one unit We are indebted to Messrs Organon Laboratories for the HMG preparations which were used as internal standards

The results obtained in 30 cases are tabulated (Table I) according to the clinical response which treatment produced Of the 12 cases in which no improvement in clinical conditions was observed four (nos 6 7 8 and 11) were still excreting at least 24 units of HMG per day after treatment in these cases the treatment had probably been ineffective in destroying the pituitary cells One of the cases showing no improvement (no 1) was excreting only four units of HMG and one showing good improvement (no 22) was excreting only one unit before treatment and in these cases the method cannot be used as an indication of change in pituitary function In six of the cases which did not improve on treatment (nos 3 4 5 9 10 and 12)

Table I

EFFECTS OF IMPLANTATION OF RADIOACTIVE MATERIAL INTO THE PITUITARY ON THE EXCRETION OF HMG AND THE CLINICAL CONDITION OF WOMEN WITH CANCER OF THE BREAST

No	Age	Material Implanted	HMG excretion (units per 24 hours) Before Implantation	After Implantation	Effect on HMG Excretion	Improvement in clinical condition patient following treatment
1	72	Y	4	5 3	?	None
2	56	Y	10	5 3	?	
3	54	Y	12	0	+++	
4	68	Y	19	0	+++	
5	47	Y	23	3	+++	
6	51	Y	24	24	0	
7	49	Au	40	24	+	
8	43	Y	45	38	0	
9	58	Au	50	1	+++	
10	37	Y	60	10	++	
11	69	Au	100	25	++	
12	69	Y	120	5	+++	
13	58	Y	14	0	+++	Slight
14	65	Au	18	33	0	
15	67	Au	30	30	0	
16	48	Y	48	2	+++	
17	62	Y	96	40	++	
18	40	Y	111	6	+++	
19	55	Au	120	23	+	
20	31	Y	192	32	+	
21	48	Au	48	55	0	Moderate
22	54	Au	1	1	?	Good
23	49	Y	10	1	+++	
24	76	Y	17	8	++	
25	63	Y	25	0	+++	
26	43	Au	30	5	+++	
27	41	Au	35	3	+++	
28	37	Y	35	2	+++	
29	54	Y	46	11	+++	
30	46	Au	60	5	+++	

the gonadotrophin excretion fell by at least 90 per cent, the tumours in these cases were presumably independent of pituitary hormones

Of the nine cases in which a good clinical response to the pituitary ablation was observed one (no 22 already mentioned) was excreting only one unit of HMG but all the other eight cases (nos 23–30) showed considerable falls in gonadotrophin excretion

The changes in gonadotrophin excretion among the nine patients showing slight or moderate improvement are less clear-cut than in

the other classes Three of these cases (nos 13, 16 and 18) showed considerable falls in HMG excretion and possibly had tumours which could be considered independent of pituitary hormones but six still had considerable gonadotrophin excretion after treatment and might have benefited by further implantation of radioactive material to destroy more of the pituitary tissue

The measurements of gonadotrophin excretion appear to give a reasonable assessment of the effect of ablation on pituitary function in those cases in which amounts of HMG sufficient for assay are excreted before treatment If only small amounts are excreted before treatment then some other test such as uptake of ^{131}I by the thyroid must be used The gonadotrophin assay is tedious taking at least a week for completion but it is the most convenient assay of the pituitary hormones

The tumours which regress following pituitary ablation in cases in which a reduction of HMG excretion occurs are not necessarily dependent on gonadotrophin itself for their growth They may need prolactin ACTH somatotrophin or possibly a hormone which is secreted by some gland under control of the pituitary such as the adrenal The simplest hypothesis is however that the tumour growth is directly dependent on one or other of the pituitary hormones HMG excretion and iodine uptake by the thyroid are probably the most convenient methods of assessing pituitary function and the effect of surgical or radiological procedures on the gland

None of the cases with HMG excretion of 80 units or more before treatment (nos 10 11 12 17 18, 19 20) responded well to the treatment although some (e g nos 12 and 18) showed marked falls in HMG excretion The numbers concerned are so few that this may be due to chance but further investigation might indicate that cases with abnormally high gonadotrophin excretion are unsuitable for pituitary ablation

REFERENCES

GREENING W P (1956) *Lancet* 2 728
LORAINE J A and BROWN J B (1954) *Acta endocr Copenhagen* 17 250

DISCUSSION

Dorfman Prof Boyland was there any possibility in the method you used of contamination of your material with oestrogens?

Boyland I have not tested it but in a number of these cases we have estimated oestrogens and found no detectable oestrogens when there was some gonadotrophin So the effect cannot always be due to oestrogen

Dorfman In these tests especially when the values go down a relatively large amount of the day s sample perhaps up to six hours or so is administered and the amount of oestrogen in the urine that can be detected by this technique is very small

Crooke We are increasingly sceptical of this method It is a bad assay from every point of view though I know it is the one that so many people use We have two colonies of mice one relatively sensitive to FSH one relatively sensitive to ICSH and we get completely different results in the two types of animal We have urines in which we have a relatively high FSH and low ICSH (urine A) and we have other urines in which the opposite occurs (urine B) as judged by the Steelman Pohley assay If we mix these in varying proportions and assay them against one another using the mouse uterus weight assay we get marked synergism with certain proportions as shown in Fig 1 We feel therefore that this assay depends upon the ratio of FSH to ICSH in the solution

Pincus Did you get a synergism on ovarian weight?

Crooke The ovarian weight of the mice in the Steelman Pohley type of assay was not affected although there was a significant increase in ovarian weight in some of the assays for total gonadotrophins in which the mice were not treated simultaneously with HCG

Boyland I must say in defence of our method that the results look quite reasonable In the case where there has been clinical improvement there is a fall where originally there was quite a reasonable excretion What is odd is that in those cases where there was a very high excretion of gonadotrophins (over 100 units in 24 hours) there has never been a really good improvement I wonder if there is any possible explanation of that

Dorfman You are using your test as a serial test in the same individual so that it might serve some useful purpose under those conditions as a rough qualitative measure

Huseby I believe it has been Dr Segaloff's experience in treating cases of human breast cancer with androgens that he gets fewer good responses in those cases that have a low pre therapy urinary excretion of gonadotrophins The majority of the favourable responses have been in those patients with a good level of urinary gonadotrophins prior to the institution of therapy and in these the level of excretion tends to decrease with treatment

Crooke We are also very concerned to find oestrogens bound to the gonadotrophins we think that will probably upset your assays

Dorfman With which of the methods do you find that?

Crooke I think you will find it in any but we have only done it with the kaolin extracts

Dorfman We tested this with the old tungstic acid method and also with an alcohol precipitation method using dialysis and washing with both alcohol and ether and it was quite free of oestrogen

Furth This discussion presupposes that the gonadotrophin is the driving force of the mammary tumour Following hypophysectomy we look on it as a measure of the residual pituitary function Uptake of iodine quantitates this even more precisely I think what is needed even

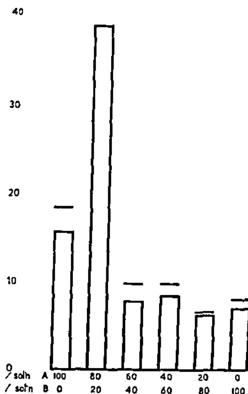


FIG 1 (Crooke) Synergism between two gona dotrophic extracts

Extracts were prepared by the kaolin acetone method from the urine of the same patient during different treatments A solu tion was prepared from each extract and groups of immature female mice were in jected with equal volumes of the solutions or of mixtures of the solutions The figure shows the mean uterine weight (and S E) for each group

(Ovarian weight showed similar changes but the mean weight in the group treated with 80 20 mixture was not significantly greater than in both groups treated with the unmixed solution)

before hypophysectomy is to find out which pituitary and other hormones drive the mammary gland Foremost is the need of a mammotrophic assay and of specific antagonists

Dorfman We certainly need as many indices as possible

Boyland I think that in different mammary tumours the hormone may be different in some cases it might be oestrogens in others an adrenal hormone or a pituitary one such as the growth hormone We cannot assume that in all cases growth is dependent on the same hormone

Furth I meant *in vitro* assays to determine on which particular hormone the tumour of a given patient depends

Dorfman The question comes up again of the accuracy of the method used to determine the values For example this method of uterine hypertrophy is usually practised in such a way that there can easily be a three or fourfold error On the other hand if you use 40 animals with about 20 on the standard and 20 on an unknown you can reduce this error to about ± 15 to 20 per cent 19 out of 20 times (Rosemberg E Smith F and Dorfman R I (1957) *Endocrinology* 61 337)

Boyland We generally use three dose levels for the unknown and two dose levels for the standards five mice are dosed at each level

GONADOTROPHINS, ANDROGENS AND OESTROGENS IN CASES OF MALIGNANT TUMOURS OF THE TESTIS

CHRISTIAN HAMBURGER

Hormone Department Statens Seruminstitut Copenhagen

Two kinds of gonadotrophic hormones may appear in the urine of patients suffering from malignant tumours of the testis viz hypophysial gonadotrophin and chorionic gonado trophin (HCG) Some testis tumours are associated with increased output of androgens others with increased oestrogen excretion The first comprehensive studies on gonadotrophins and testis tumours were reported by Ferguson and co workers (1931) Since then numerous reports and many systematic investigations have appeared The pertinent literature has been carefully reviewed by Twombley (1944) Reference is also made here to the important monograph of Sorba (1946)

In Denmark Bang (pathologist) Nielsen (radiologist) and the author (endocrinologist) have tried to throw light on the correlation between the morphology clinical course and endo crine functions of malignant testis tumours (Hamburger 1933, Bang Hamburger and Nielsen 1935 Hamburger Bang and Nielsen 1936 Hamburger 1941 Hamburger and Godtfredsen 1941 Hamburger 1947) Between 400 and 500 cases of malignant testis tumours were examined the main results of the investigations being as follows some of the tumours and their metastases may contain trophoblast cells these tumours are extremely malignant and radioresistant, and they give rise to excretion of HCG Other testicular tumours are associated with increased excretion of hypo physial gonadotrophin that is found in small quantities in normal urines and usually designated as follicle stimulating hormone (FSH)

The classification of the testis tumours has always been very difficult and confusing The Danish pathologist Teilum (1946) has proposed a classification that both from theoretical and practical points of view seems to be very useful Table I shows the most common malignant testis tumours their places in Teilum s system and their endocrine functions

Table I

TESTIS TUMOURS
CLASSIFICATION AND HORMONE PRODUCTION
(Teilum 1946)

Type of tumour	*Hormone production*		
	Chor Gon	*Oestrogen*	*Androgen*
SEMINOMA SERIES			
Seminoma testis	0	0	0
Epithelioma adenopapilliferum	(+)	?	0
Chorionepithelioma	+	(+)	0
ANDROBLASTOMA SERIES			
Androblast diff (Leydig cell tumour)	0	0	+
Androblast tubulare lipoides (Sertoli cell tumour)	0	+	0

The material to be presented here comprises 436 patients in whom urinary hormone analyses have been carried out before and/or after the removal of the primary tumour More than half of the tumours were seminomas then come the chorionepitheliomas (designated by Bang as epithelioma mixtum) followed by the adenopapilliferic epitheliomas which seem to cover Bang s small cystic mixed tumours Other forms of testis tumours including interstitial cell tumours sarcomas etc are numerically of minor importance

Technique

The gonadotrophic hormones were assayed either in tannic acid precipitated urines or in untreated or diluted specimens of urine The kind of gonadotrophin present was determined

in most cases on the basis of the morphological changes in the ovaries of immature mice With the doses given the hypophysial gonadotrophin usually stimulates the follicular growth, while HCG gives rise to the formation of corpora lutea and blood filled follicles In many cases immature female rats were used for the identification and quantification of the gonadotrophins The mean weights of ovaries and uteri were measured and the resulting dose response curves compared *with those of known gonadotrophins* (HCG and hypophysial gonadotrophin from the urine of postmenopausal women)

The androgenic substances were in most cases measured biologically by the capon comb technique or chemically as total neutral 17 ketosteroids The oestrogenic substances were assayed by the Allen Doisy technique in spayed mice

Results

The great majority (86 per cent) of the patients were young or middle aged (20–49 years) The results of the gonadotro phin assays are summarized in Table II In 123 of the cases

Table II

EXCRETION OF GONADOTROPHINS IN 436 CASES OF TESTIS TUMOURS

Gonadotrophins	*Number of Patients*		
	Total	*Per cent*	
0(<50 mouse units)	123	28	75
<50 m u (traces)	55	13	
≧50 m u hyp gon	147	34	
Chor gon	87	20	22
Chor gon ⇄ hyp gon	8	1 8	
Chor gon +hyp gon	3	0 7	
Type uncertain	13	3	

gonadotrophins could not be determined by the technique used, i e the amount was below 50 mouse units per day In 55 cases small amounts were found, but below 50 m u per day, i e it was not shown that the excretion exceeded the normal

excretion A definitely increased excretion was obtained by one or more examinations of urines from not less than 147 patients (34 per cent of the cases) Ninety eight of the patients (22 5 per cent) excreted HCG on one or several occasions In rare instances when urines were examined serially for long periods of time the first urines might contain HCG before the ablation of the primary tumour and later on hypo physial gonadotrophin Or in other cases the reverse order was observed some time after surgery the urines contained hypophysial gonadotrophin but later on when metastases containing trophoblast like cells had developed excretion of HCG commenced Quite exceptionally it was possible to demonstrate the simultaneous occurrence of both kinds of gonadotrophin

One of three such cases was examined very carefully and has been published previously (Hamburger 1941) It was a young man who had been hemicastrated for a malignant testis tumour containing trophoblast like cells In the course of the first six months after the operation, increased excretion of hypophysial gonadotrophin (FSH) occurred in some of the specimens of urine examined Somewhat later when metastases had developed several specimens of his urine contained gonadotrophin that according to the histological ovarian changes and the dose response curves in immature rats was strikingly similar to pregnant mare s serum gonado trophin and differed markedly from hypophysial and chorionic gonadotrophins However by injecting rats with various doses of a mixture of postmenopausal hypophysial gonado trophin and chorionic gonadotrophin in the proportion 1 rat unit to 4 i u exactly the same uterine and ovarian dose response curves were obtained From this and many other assays *it was calculated that the urine contained 80 rat units* of hypophysial gonadotrophin and 300 i u of HCG per 24 hr volume The simultaneous excretion of the two gonado trophins seems to occur only when the HCG production by the chorionepithelioma metastases commences rather suddenly in a patient in whom a hyperproduction of hypophysial

gonadotrophin is present Probably it takes some time before the hypophysial hyperactivity has been suppressed by the circulating HCG It is reasonable to assume that exactly the same phenomenon would occur if a castrated woman became pregnant!

The relation between androgen and gonadotrophin excretion is shown in Table III The average normal excretion in middle aged men is about 40 i u per day, the lowest normal limit being 20 i u The patients with testis tumours

Table III

EXCRETION OF ANDROGENS IN 137 CASES OF TESTIS TUMOURS

Group acc to excretion of gon	*No of patients*	*Mean daily excretion i u ±s e*	*Number of patients excreting*			
			<20 i u /24 hr		*≧20 i u /24 hr*	
			Abs	*%*	*Abs*	*%*
0(<50 m u)	42	21 4±2 96	23	55	19	45
≧50 m u hyp	45	14 4±2 08	35	78	10	22
Chor gon	43	17 3±2 08	25	58	18	42
Type uncertain	7	25 1±8 51	4	57	3	43

have on the average a low androgen production and it is lowest in the 45 patients who had an increased excretion of hypophysial gonadotrophin This finding supports the assumption that the increased excretion of hypophysial gonadotrophin is secondary to a low androgen formation The androgen excretion is somewhat higher in patients with HCG production and 42 per cent as against 22 per cent, have an excretion within the normal limits The most reasonable explanation of this finding is that the HCG has stimulated the androgen formation of the remaining testis or that of the adrenal cortex It was found that there is no correlation between the quantities of HCG and of androgens and it is therefore not likely that the tumour cells that produce HCG (and sometimes also oestrogens cf below) also produce androgens

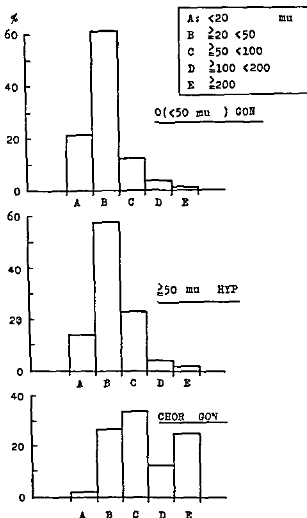


FIG 1 Relation between excretion of gonadotrophins and oestrogens in cases of testis tumours (mu = mouse units)

The total neutral 17 ketosteroids that are more dependent on the adrenocortical than on the testis function were some what lower in the cases with increased gonadotrophin excretion than in cases without increased excretion

Sorba (1946) was of the opinion that the testis seminomas themselves produce hypophysial gonadotrophin, but our findings go against this assumption Out of 12 patients hemicastrated for seminoma and in whom no metastases had developed, not less than 10 had, on one or more occasions, an elevated excretion of hypophysial gonadotrophin and 2 of 7 patients with seminoma metastases excreted below 50 m u gonadotrophin per day

The excretion of biologically active oestrogenic substances was measured once or repeatedly in 206 of the cases An excretion above 100 m u per day is definitely abnormal Such high excretion occurred in only 5 out of 137 patients without increased gonadotrophin excretion or with increased excretion of hypophysial gonadotrophin, but in 22 out of 59 patients excreting HCG The percentage distribution of low, normal, and high oestrogen excretion in the three groups of patients is shown in Fig 1 It must be regarded as an established fact that testis tumour tissues producing HCG also possess the ability to produce oestrogens

Conclusions and Summary

The main conclusions of the present investigations are the following

Independent of the histological structure of the tumour, patients who suffer from or who have been operated on for malignant testis tumours may excrete abnormally high amounts of hypophysial gonadotrophin The excretion is not influenced by the removal of the primary tumour nor by the development of metastases It never exceeds the amounts found in urines after castration or in postmenopausal women The increased production of hypophysial gonadotrophin is probably caused by a low androgen production This may be due to a reduction of the total amount of normal testicular

tissue from the development of the tumour in one testis or from the hemicastration Toxic effects of the tumour tissues and repeated X ray treatments may also play a part The finding of increased excretion of hypophysial gonadotrophin is too unspecific to have any diagnostic value in cases of testis tumours

In cases where the primary tumour or the metastases contain cells similar to trophoblast cells (e g chorionepith eliomas) chorionic gonadotrophin (and increased amounts of oestrogens) will usually be present in the urine The excretion of chorionic gonadotrophin reflects the amount of tumour tissue present and the hormones are unquestionably produced by the trophoblast like syncytial cells themselves The presence of chorionic gonadotrophin in the urine of a male subject is pathognomonic of a malignant testis tumour (or of an extragenital chorionepithelioma) and as the tumours producing chorionic gonadotrophin are extremely malignant and radioresistant the presence of this hormone is a very bad prognostic sign The high malignancy of the chorion epitheliomas of the testis contrasts markedly with the good prognosis of chorionepitheliomas of the uterus

REFERENCES

BANG F HAMBURGER C and NIELSEN J (1935) *Bull Ass franç Cancer* 24 418
FERGUSON R S DOWNES H R ELLIS E and NICHOLSON M E (1931) *Amer J Cancer* 15 835
HAMBURGER C (1933) Studies on gonadotropic hormones from the hypophysis and chorionic tissue Copenhagen Levin and Munksgaard
HAMBURGER C (1941) *Acta path microbiol scand* 18 457
HAMBURGER C (1947) *J Endocrin* 5 xxiv
HAMBURGER C BANG F and NIELSEN J (1936) *Acta path microbiol scand* 13 75
HAMBURGER C and GODTFREDSEN E (1941) *Acta path microbiol scand* 18 485
SORBA M (1946) *Helv chir acta* 13 1 Suppl 2
TILLUM G (1946) *Acta path microbiol scand* 23 242 252
TWOMBLEY G H (1944) *Surgery* 16 181

[Discussion after this paper was postponed until after the short communication by Dr Crooke —EDS]

Short Communication

FOLLICLE STIMULATING HORMONE IN THE URINE OF PREGNANT WOMEN

A C Crooke W R Butt Joyce D Ingram
and Brenda P Round

Department of Clinical Endocrinology
The Birmingham and Midland Hospital for Women

Dr Hamburger's methods for measuring urinary follicle stimulating hormone (FSH) and human chorionic gonadotrophin (HCG) are very ingenious and clinically useful They are neither specific nor sensitive enough however to measure the relatively low concentration of FSH which is normally present in the urine of pregnant women and probably also of patients with tumours secreting HCG We thought it might be of interest to describe a method which we have developed in Birmingham and which will do this It is suitable for statistical analysis but unfortunately we have no data yet on patients with gonadotrophin secreting tumours

This assay for FSH employs the augmentation principle developed by Steelman and Pohley (1953) for rats and by Brown (1955) in our department for mice Immature female mice are injected with FSH together with an excess of luteinizing hormone which is given as HCG The HCG causes a slight increase in ovarian weight and much larger doses cause no further enlargement but when FSH is added there is a pronounced additional increase in ovarian weight This principle is at variance with the findings of Hamburger (1941) who showed that HCG causes a slight progressive increase in the ovarian weight of immature rats as the dose is increased With very large doses there is a further rapid increase in weight This response is shown in Fig 1 (Emmens 1950) In contrast with these observations we found that there was no significant difference in the ovarian weight of our mice when given HCG in doses varying between 20 and 1 000 i u We have used 40 i u of HCG in all routine assays of FSH and we give the control mice 1 and 3 mg respectively of FSH as the British Standard HMG20A The response is shown in Fig 2

It is likely that in assaying extracts of urine from pregnant women for FSH the extracts will be contaminated with high concentrations of HCG There may be considerably more than 1 000 i u per mouse and it becomes necessary to effect at least a partial separation of FSH and HCG as a precaution against overdosage

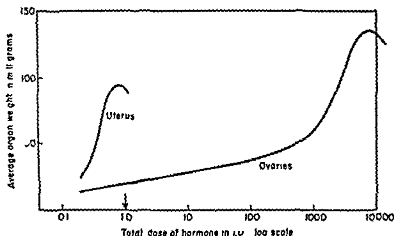


FIG 1 Average uterine and ovarian weight curves obtained in immature rats after treatment with chorionic gonadotrophin

with HCG We have succeeded in attaining this by the combination of two well known extraction procedures the benzoic acid precipitation method of Katzman and Doisy (1934) and the kaolin tricalcium phosphate method of Crooke and co-workers (1954) In order to investigate the precipitation of gonadotrophins from urine by

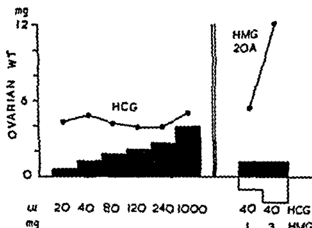


FIG 2 Synergism between HCG (benzoic acid) and HMG20A

benzoic acid a series of experiments was performed The urine of pregnant women was first extracted by the benzoic acid method and the residual urine was then extracted by adsorption on kaolin The benzoic acid extract was assayed against the kaolin extract by the method which depends on the increase in weight of the whole prostate of the rat (Loraine 1950) In three experiments it was found that 75 80 and 91 per cent respectively of the activity was in the benzoic acid fraction This assay is relatively sensitive to HCG but it is not claimed to be specific for it It shows however, that roughly four fifths of the active material had been precipitated by benzoic acid

Three samples of benzoic acid extract of urine from pregnant women were assayed against International Standard CG by the rat prostate method They were then injected into immature female mice and produced the same ovarian response as that observed previously This shows that the amount of FSH in the benzoic acid extracts was negligible although FSH was subsequently extracted from the residual urine In order to determine the amount of FSH extracted by benzoic acid more accurately it was decided to use urine from postmenopausal women containing a relatively high concentration of FSH Two samples were used Each was extracted as before and the benzoic acid extract was assayed against the kaolin extract using the procedure described previously for the assay of FSH In the first 9 7 per cent (95 per cent fiducial limits 5–15 per cent) and in the second 7 0 per cent (95 per cent fiducial limits 2 7–11 0 per cent) of the activity was found in the benzoic acid extract compared with the kaolin extract It is fair to assume therefore that probably less than one tenth of the FSH is lost in the benzoic acid extract

In all subsequent experiments with pregnant women the urine was first treated with benzoic acid and the residue was extracted by the kaolin method and fractionated by adsorption on tricalcium phosphate into what we have previously described as the GA and GB fractions It was hoped that FSH would be found only in the GA fraction but in order to confirm this both GA and GB fractions were assayed for FSH against the British Standard HMG20A Active material was always found in the GA fraction but occasionally some activity was also present in the GB fraction This method of fractionation on tricalcium phosphate is not therefore very satisfactory The assays also showed that there was no significant difference in the slopes of the dose response curves of the Standard HMG20A and of the unknown materials An example is shown in Fig 8 This demonstrates that FSH from the urine of pregnant women behaves in the same way as FSH from postmenopausal

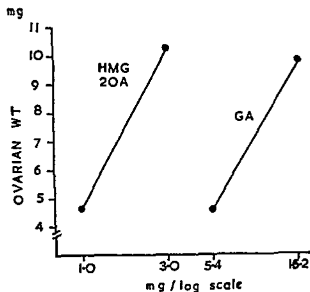


Fig 3 FSH in pregnancy

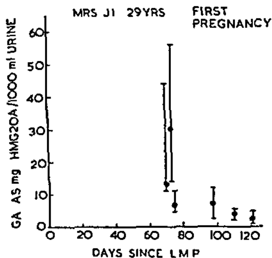


Fig 4 Amount of FSH excreted by a woman aged 29 at different stages of pregnancy

women in our assays which suggests that the two substances are physiologically identical

Finally we have some preliminary results which show the amount of FSH excreted by a woman aged 29 at different stages of pregnancy They are given in Fig 4 and are expressed as mg of the Standard HMG20A in the GA fraction per litre of urine They are all based on four point assays and the 95 per cent fiducial limits are shown It appears that the excretion of FSH rises in the early months of pregnancy and falls later like HCG (Butt et al 1957)

REFERENCES

BROWN P S (1955) J Endocrin 13 59

BUTT W R CROOKE A C INGRAM JOYCE D and ROUND BRENDA P (1957) J Endocrin 16 107

CROOKE A C BUTT, W R INGRAM JOYCE D and ROMANCHUCK LUDMILA E (1954) Lancet 1, 379

EMMENS C W (1950) Hormone Assay New York Academic Press Inc

HAMBURGER C (1941) Acta path microbiol scand 18 457

KATZMAN I A and DOISY E A (1934) J biol Chem 106 125

LORAINE J A (1950) J Endocrin 6 319

STEELMAN S L and POHLEY F M (1953) Endocrinology 53 604

DISCUSSION

Pincus Do you think the oestrogens come directly from the chorionic tissue or is there a possibility that they originate from other tissues by stimulation with chorionic gonadotrophin Dr Hamburger?

Hamburger Admittedly we do not know from which cells the oestrogens originate in the extremely polymorphous oestrogen producing testis tumours In view of the fact that they contain trophoblast like cells and that chorionic tissue can produce oestrogens it is reasonable to assume (but it is not proved) that these cells are the source of the oestrogens

Pincus Is there any sign of increased activity of the interstitial cells of the testis in these cases?

Hamburger It may be difficult to identify interstitial cells in chorionepitheliomas of the testis and their metastases It is very likely that in cases of high gonadotrophin production the Leydig cells in the other testis would show signs of hyperactivity I do not recall whether the pathologists have described the appearance of the interstitial tissue in the remaining testis

Boyland Have you tried any chemotherapeutic agents on the seminomas? There is a phenylalanine nitrogen mustard which is claimed to have a beneficial effect on them It would be interesting to see if you could correlate changes in hormone secretion with clinical changes following treatment

Hamburger It would be very interesting but to my knowledge the treatment in the cases I have hormone analysed has consisted of surgery and radiotherapy only

Finkelstein We have had a few cases of chorionepithelioma in the uterus with high secretion of chorionic gonadotrophin—a few millions—but there was no high oestrogen excretion

Hamburger We have not investigated the oestrogen excretion in cases of chorionepithelioma of the uterus Your observation is highly interesting and it might indicate that there is a fundamental difference between the chorionepitheliomas of uterus and testis or that the oestrogens in the testis chorionepitheliomas originate from other cells than the trophoblast like cells

Finkelstein In the testis we had one case where we found high oestrogen excretion associated with the high chorionic gonadotrophin

Pincus I think it is true that normally if you administer chorionic gonadotrophin to a male you get increased oestrogen excretion and what I was trying to get at was whether this was actually a stimulation of the steroidogenic tissue of the testis rather than direct production by the chorionepithelioma tissue

Dorfman It has been very definitely established that at least in the adult testis gonadotrophin will increase both androgen production and oestrogen The oestrogen shows a somewhat larger percentage increase as compared to that found for the androgens This is only true of the adult testis in children it is almost impossible and it is similarly difficult in old age The ovary does not respond very well at least not with an increase in 17 ketosteroids

Furth How can luteinization alone be characteristic of human chorionic gonadotrophin? Hypophysial gonadotrophins are also luteinizing

Hamburger If we administer sufficiently high amounts of the hypophysial gonadotrophin present in normal or castrate urines we certainly do get luteinization in the ovaries of immature mice and hypophysectomized rats But with the quantities used in the practical tests we obtain a pure FSH reaction in immature mice treated with castrate urine I am sorry if I have not expressed myself clearly enough I will not maintain that the mere presence of some corpora lutea in the ovaries of the immature mice indicates that the gonadotrophin is of chorionic origin Histological examinations of the mouse ovaries reveal characteristic differences between hypophysial gonadotrophin in castrate or post menopausal urines and chorionic gonadotrophin Furthermore in many of our cases complete uterine and ovarian dose response curves for immature female rats were established and such curves enable us to distinguish between the two kinds of gonadotrophin

Pincus It is very interesting that you had only three cases from this large group in which there was evidence for a mixture If your method was able to distinguish the mixture even in the case of very small amounts then you should have seen it in larger numbers That points to the fact that there is not much chance of confusion with the methods used

Luft It might be of some interest in this connexion to mention that

in some laboratories the effect of large doses of chorionic gonadotrophin on the excretion of steroid hormones has been studied in patients with endocrine disturbances The dosage has been 3 000–6 000 units given daily for two or more days Such a test was made in one of our patients by Dr Tillinger The patient was a woman of 20 who was lacking pubic and axillary hair growth and who at examination turned out to have an atrophic vagina At operation the gonads were found to be testicles The sex chromatin had a male pattern The testicles were removed by the surgeon Administration of 6 000 units of chorionic gonadotrophin for two consecutive days before castration was followed by an increase in the urinary excretion of oestrogens To our surprise the same finding was obtained after castration There was also some increase in the urinary 17 ketosteroids on stimulation with gonadotrophin before operation

Dorfman Did you administer the gonadotrophin intravenously?

Luft No this was administered intramuscularly

Dorfman There are a few groups that have started to use this as a functional test but many people have thought that they would like an intravenous preparation Is such a preparation available in Sweden?

Luft The preparation of chorionic gonadotrophin used in our country is Gonadex manufactured by the Leo Company To my knowledge this cannot be administered intravenously

Crooke Even though the material is crystalline that does not mean it does not contain FSH

Pincus Is there any evidence of pituitary dependence of these tumours in man?

Hamburger I do not think so The possibility has however been discussed that an increased production of hypophysial gonadotrophin might be the primary factor in the development of seminomas of the testis If this were the case it would be difficult to explain the low androgen excretion Furthermore we would expect these tumours to be more frequent in the older age groups in which an increased gonadotrophin production is the rule rather than in young and middle aged males

Luft Hypophysectomy has been tried in some patients with seminoma and cancer of the testes The results have been disappointing and as far as I know no favourable response has been seen We may have to take it into consideration that the hypophysectomies were not all complete We had a surprising experience in a girl of 18 suffering from a chorionepithelioma or malignant mole The tumour was first diagnosed in connexion with a septic abortion and the uterus was removed Two months later X ray films of the lungs showed two small metastases The excretion of chorionic gonadotrophin was up to 70 000 units per day Hypophysectomy was performed about four months after removal of the uterus The excretion of chorionic gonadotrophin dropped from 70 000 units per day to zero within a few months at which level it then remained The girl died five months after hypophysectomy from a second disease (an arteriovenous aneurysm) At autopsy there were no signs of chorionepithelioma anywhere in the body

Gardner The statement has been made that in normal trophoblastic

tissues the cytotrophoblast may be associated with the chorionic gonadotrophin production and the syncytial trophoblast with the oestrogen production Is there any difference in the cytology of these tumours which produce oestrogens in contrast to those which do not that might homologize with the assumption that has been made with respect to the cellular differentiation of hormone production in the placental tissues?

Hamburger It is a very important question but it may be difficult to find appropriate cases because in most of the testis chorionepithelioma cases there is a high excretion of chorionic gonadotrophin and oestrogens simultaneously

Dorfman Dr Hamburger I think one important constituent that should be looked for now in view of our working hypothesis of the formation of the androgens and the oestrogens is the pregnanediol in the urine Have you any data on pregnanediol excretion?

Hamburger We have not made pregnanediol determinations The investigations reported here were finished about seven years ago It is our intention to take them up again using more refined techniques for the steroid determinations including fractionated 17 ketosteroid analyses chemical determinations of the individual oestrogens and also pregnanediol assays

Dorfman You seem to get a tremendous number of these cases in Denmark In view of the fact that you are taking up this work again perhaps it might be wise to study the biosynthetic potentials of these tissues with radioactive compounds

Hamburger I hope we will be able to contribute to such investigations I do not think we have relatively more cases of malignant testis tumours in Denmark than in other countries the 500 cases were collected over about 18 years and because Denmark is a small country (of about four million inhabitants) we have been able to maintain close contact with all the radium stations and the larger hospitals

Scowen That is not really a large proportion of cases

INTERSTITIAL CELL TUMOURS OF THE MOUSE TESTIS
STUDIES OF TUMORIGENESIS, DEPENDENCY AND HORMONE PRODUCTION*

ROBERT A HUSEBY

Department of Surgery University of Colorado School of Medicine Denver

Tumorigenesis

PRIOR to the initiation of these studies, interstitial cell tumours of the mouse testis had been induced only by treating genetically susceptible mice with large doses of oestrogenically active substances, either steroidal or non steroidal (for review see Andervont, Shimkin and Canter, 1957) Gardner (1948) had suggested that the mode of action of the oestrogens was to increase the pituitary elaboration of luteinizing hormone (LH) which then stimulated the interstitial cells excessively The present experiments were undertaken in a search for other means of inducing this type of neoplasia and in an attempt to determine if in fact excessive stimulation with pituitary LH did play a pivotal role in their genesis

It had been shown by Smelser (1933) that ovaries would produce significant amounts of oestrogen when homotransplanted to male guinea pigs that had been made cryptorchid surgically A small group of BALB/c male mice were rendered cryptorchid and two ovaries were isotransplanted to their axillary tissues after preliminary studies had shown that as in guinea pigs, such ovaries produced easily detectable amounts of oestrogen When a number of testicular interstitial cell tumours developed (Huseby and Bittner 1952) a more

* This work was supported in part by res arch grants made to the University of Colorado by the Damon Runyon Memorial Fund



Fig. 1. Low power view of a testis of a BALB/c mouse 4 months of age that had been rendered cryptorchid surgically at 1 month of age. A considerable portion of this small testis is composed of very abnormal interstitial cells. All tubular elements have been calcified (× 40).



FIG 2 Higher power view of abnormal interstitial cells comprising nodule pictures in Fig 1 Because of the degree of cellular pleomorphism and the presence of mitotic figures this was classified as an interstitial cell tumour (× 270)

extensive series of investigations was undertaken to determine the relative roles of mild oestrogenization and of the intra abdominal position of the testis upon tumorigenesis and to attempt to evaluate the endogenous production of pituitary LH under the various experimental conditions employed

To obtain mild exogenous oestrogenization stilboestrol (3 4 di *p* hydroxyphenylhex 3 ene) was incorporated in a semisynthetic diet in a concentration of 0 2 µg per gram (BALB/c mice consume 2 to 2 5 g of this diet per day) Studies revealed that at this dosage level one month old animals tolerated treatment well for periods of many months and that little if any histological alteration of the testes resulted during a three month test period The seminal vesicles of intact mice were only mildly reduced in size and such mice remained fertile as shown by breeding tests employing non oestrogenized females Castrate males responded to injections of graded doses of testosterone quantitatively the same as did castrates ingesting the same diet lacking stilboestrol

The results of these experiments are summarized in Table I It is evident that relatively mild oestrogenization whether from ovarian secretion or stilboestrol ingestion induces tumour formation in a high percentage of cryptorchid testes but is considerably less tumorigenic for testes that remain in the scrotum Long residence within the abdominal cavity in itself appears to exert a definite tumorigenic effect Of the 15 animals in which the surgical procedure employed resulted in the majority of the testes remaining well up in the abdominal cavity for the remaining life of the animal seven developed testicular lesions that we felt must be classified as interstitial cell tumours (Figs 1 and 2) In all instances these were of small size none enlarged the already small cryptorchid testis though in several instances they replaced much of it All however, were composed of cells that had morphological characteristics identical to those cells making up the large interstitial cell tumours occurring in the other treatment groups In the testes of each of the other eight

animals of this group, areas of interstitial cell hyperplasia were evident that occasionally were as large as the previously described areas of tumour but which were composed of morphologically more normal appearing interstitial cells It is apparent that the degree of cryptorchism is a factor in the production of these changes for in another group of 16

Table I

DEVELOPMENT OF INTERSTITIAL CELL TUMOURS OF THE TESTIS IN BALB/c MICE

	Treatment	*No tumorous/ No animals*	*Average tumour age (months)*	*Seminal vesicular size after 3 months treatment*	*Interstitial cell morphology after 3 months treatment*
I	10% stilboestrol pellet	12/14 (86%)	11 5	Less than 50% of normal (squamous metaplasia)	Abnormal hyperplasia with brown degeneration
II	Cryptorchid with transplanted ovaries	27/35 (77%)	10 3	Less than 50% of normal (variable)	Slightly atrophied
III	Cryptorchid on stilboestrol diet	18/22 (82%)	20 1	80% of normal	Essentially normal
IV	Stilboestrol diet only	8/24 (33%)	19 2	80% of normal	Essentially normal
V	Cryptorchid only	7*/15 (47%)	22 9	Normal	Hypertrophied
VI	Cryptorchid only (incomplete)	2*/16 (12 5%)	22 6	—	—

All treatments were begun when the mice were 4 to 6 weeks of age and were continued for the remainder of their lives Fifty untreated males were observed as controls None developed grossly detectable testicular tumours and histological examination of the testes of half of them failed to reveal either small areas of tumour or significant areas of interstitial cell hyperplasia

* All of these tumours were of small size none enlarged the small cryptorchid testes.

animals a surgical procedure was used that resulted in most of the testes remaining near the inguinal ring In this situation the tubular changes were less pronounced only two small histologically typical tumours developed and in only three of the other animals were significant areas of hyperplasia noted

In these studies there was no obvious correlation between the appearance of the non tumorous interstitial cells and the

frequency of tumour development An abnormal interstitial cell hyperplasia associated with extensive brown degeneration is described by most investigators studying the induction of interstitial cell tumours by means of heavy oestrogenization This type of change did not occur in the testes of the mildly oestrogenized mice in these experiments Not only was it not present after three months of treatment but also examination of the contralateral testes of those animals that did not develop bilateral tumours usually revealed a predominance of essentially normal appearing interstitial cells Similarly the size of the seminal vesicles noted after three months of treatment did not seem to correlate with the incidence of tumours

It would appear that the evidence obtained in these studies argues against the hypothesis that oestrogen induced interstitial cell tumours of the mouse testis result from an over stimulation of the normal interstitial cells by endogenously produced LH This does not mean that LH stimulation is not necessary for tumour production (the experiments of Ely (1953) certainly suggest that it may be) but rather that excessive stimulation by LH is not the mechanism by which these neoplasms are produced It would appear furthermore that the intra abdominal position definitely favours tumour development Whether this effect of cryptorchism is a systemic or a local one is presently under investigation

In order to investigate the role of LH in interstitial cell tumorigenesis further the following experiment was carried out Intact male BALB/c mice were treated in one of several ways for a period of four months their testes were then minced and implanted subcutaneously into castrate male BALB/c animals bearing 10 per cent stilboestrol pellets and these recipients were observed for the development of interstitial cell tumours at the site of the implants The treatments employed and the results obtained are summarized in Table II

On the basis of these data it would seem that treatment with a high dose of stilboestrol for only four months definitely favours the development of interstitial cell tumours when

Table II

DEVELOPMENT OF INTERSTITIAL CELL TUMOURS IN CASTRATE BALB/c MICE BEARING 10% STILBOESTROL PELLETS AND IMPLANTED WITH MINCED TESTES FROM BALB/c MALE ANIMALS TREATED IN VARIOUS WAYS FOR FOUR MONTHS

Group	Treatment of donors	No donors	No recipients	Recipients developing tumours	Average tumour age (days)*	Average NTD (days)*
I	10% Stilboestrol	8	37	20 (54.1%)	28[illegible]	410
II	10% Stilboestrol and pituitary LH	6	27	18 (66.7%)	244	[illegible]96
III	Pituitary LH	11	60	10 (16.7%)	376	346
IV	Untreated	12	60	10 (16.7%)	438	3[illegible]

LH treatment consisted of daily injections of Armour's sheep LH No 227-80
Excluded from the compilation of group I are six recipients transplanted with a testis that was grossly tumorous all animals developed tumours within a six week period

* Calculated from time of implantation of testicular mince
NTD = non tumour death

the interstitial cells are then transplanted into oestrogenized recipients Since the interstitial tissue is being transplanted into an environment which in itself is capable of inducing tumour formation in normal interstitial cells it might be possible that the effectiveness of this relatively short treatment period with stilboestrol prior to transplantation resided solely in the fact that such treatment causes a hyperplasia of the interstitial cells More interstitial cells would therefore be present in each of the grafts and thus the statistical probability of a tumour developing from one or more of the transplanted interstitial cells would be increased That this is not the correct interpretation seems evident from the fact that treatment with pituitary LH alone did not increase the frequency with which the tumours developed in the recipient animals, although it did cause a hyperplasia of the interstitial cells to essentially the same degree as that seen in the stilboestrol treated animals There is however a major difference in *the two types of treatments With stilboestrol* the interstitial cells in the resultant hyperplasia have a very abnormal morphology whereas with LH treatment the

interstitial cells appear normal although hyperfunctional, that is their nuclei are somewhat larger than normal and they have an increased amount of a highly vacuolated cyto plasm It would appear most probable therefore that by employing this technique one is actually studying factors involved in interstitial cell tumorigenesis *per se* and not merely quantifying the number of interstitial cells present in the explants

The data obtained to date do not clearly define the role if any of LH in interstitial cell tumorigenesis It seems fairly clear that an increased stimulation for a period of four months with sheep pituitary LH by itself had little or no tumorigenic effect (comparison of groups III and IV) However due to the fact that many animals of group II died relatively young as indicated by the lower average age at death of the non tumorous animals in this group it cannot be said with certainty that LH treatment combined with oestrogenization does not augment tumorigenesis

Tumour Dependency

That certain malignant neoplasms are not completely autonomous but rather depend to some degree upon stimuli from the host for their continued progressive growth is a fairly new concept in the field of oncology and to date much of the information supporting this concept comes from clinical medicine It has been known for some time that interstitial cell tumours of the mouse testis induced by oestrogen adminis tration generally require that the recipient animals also be oestrogenized if isotransplants are to grow progressively (for review see Andervont Shimkin and Canter 1957) Thus it would appear that these tumours at least during their initial transfer generations are dependent according to the present day concept and a few inquiries into the details of the characteristics of this dependency have been reported (Andervont Shimkin and Canter 1957 Huseby, 1954 Jull 1954) The present studies were undertaken in an attempt

to obtain additional basic information regarding this interesting and important phenomenon

General pattern of dependency

Early in the course of these investigations it was hoped that by studying the general dependency characteristics of a number of different interstitial cell tumours over several transfer generations, a pattern would evolve that would give some insight into the individual factors responsible for supporting the growth of these tumours To date seven transfer lines have been studied rather intensively, and the picture that has evolved appears to be most complex and not readily interpretable, as is apparent from an inspection of the results summarized in Table III It is evident that variations

Table III

GENERAL PICTURE OF TUMOUR DEPENDENCY IN SIX TRANSPLANTABLE LINES OF TESTICULAR INTERSTITIAL CELL TUMOURS INDUCED IN BALB/c MICE

Tumour	*Transfer Generation*	*Most rapid growth*	*Less rapid growth*	*Inconsistent or no growth*
157220 Line B	4–15	Oestrogenized ♂♂ and ♀♀ (heavily)	—	Oestrogenized ♂♂ and ♀♀ (mildly) Intact ♂♂ and ♀♀ Castrate ♂♂ and ♀♀ Intact ♂♂+TP
157220 Line A	7–10	Oestrogenized ♂♂ Intact ♀♀	Castrate ♂♂ and ♀♀ Intact ♂♂+TP	Intact ♂♂
4061	2–7	Oestrogenized ♂♂	Intact ♂♂ and ♀♀	Castrate ♂♂ and ♀♀
4092	2–9	Oestrogenized ♂♂ Castrate ♂♂ and ♀♀	—	Intact ♂♂ and ♀♀ Intact ♂♂+TP
166189	7–9	Oestrogenized ♂♂ Intact ♀♀	Castrate ♀♀	Intact ♂♂ Castrate ♂♂ Intact ♂♂+TP
164041	4 and 5	Oestrogenized ♂♂ Intact ♀♀ Castrate ♂♂ and ♀♀	Intact ♂♂	—

TP = treatment with testosterone propionate 2 5 mg twice weekly
These experiments were continued only until the animals in the most rapid growth groups had sizable tumours at which time all animals on the experiment were killed It is possible therefore that had the experiments been continued longer growth might have occurred in some animals in the "inconsistent or no growth" groups

in the degree of dependency appeared in the different tumour lines relatively early in the process of serial transplantation and that in the case of tumour 157220 two distinct lines with very different characteristics developed Treatment with stilboestrol routinely elicited as rapid growth of all tumour lines as did any other type of endocrine alteration Beyond this, however there appears to be no consistency in the patterns of hormone dependency Thus tumours 157220 Line A and 166189 grew more rapidly in intact female recipients than they did in castrate females while the reverse situation obtained in line 4092 Similarly, although line 4061 grew rather slowly in intact females it grew essentially as well in intact males yet did not grow in either castrate male or female recipients Just as peculiar treatment of intact males with large doses of testosterone propionate induced growth of 157220 Line A while the same treatment was entirely in effectual in the case of three other tumour lines

It seems most probable on the basis of these findings that there are several endocrine factors influencing the growth of these dependent tumours During tumorigenesis and sub sequent serial transplantation the general tendency appears to be for a tumour to lose or reduce its requirement for one or more of these stimulants but these losses do not appear to occur in any given sequence so that lines evolve that have quite different dependency characteristics

The general experience of interstitial cell tumours decreas ing in dependency upon serial transplantation was well illustrated in one tumour line studied (4658—results not included in Table III) During the second transfer it grew only in oestrogenized recipients but by the third generation it showed some tendency to grow in intact females In the fifth transfer generation it grew readily in castrate males and females as well as in oestrogenized males and intact females, and by the seventh transfer it would grow in intact males also Quite in contrast however 157220 Line B has retained full dependency through 15 transfer generations over a period of time in excess of four years Subcutaneous grafts of this

to obtain additional basic information regarding this interesting and important phenomenon

General pattern of dependency

Early in the course of these investigations it was hoped that by studying the general dependency characteristics of a number of different interstitial cell tumours over several transfer generations a pattern would evolve that would give some insight into the individual factors responsible for supporting the growth of these tumours To date seven transfer lines have been studied rather intensively, and the picture that has evolved appears to be most complex and not readily interpretable as is apparent from an inspection of the results summarized in Table III It is evident that variations

Table III

GENERAL PICTURE OF TUMOUR DEPENDENCY IN SIX TRANSPLANTABLE LINES OF TESTICULAR INTERSTITIAL CELL TUMOURS INDUCED IN BALB/c MICE

Tumour	*Transfer Generation*	*Most rapid growth*	*Less rapid growth*	*Inconsistent or no growth*
157220 Line B	4–15	Oestrogenized ♂♂ and ♀♀ (heavily)	—	Oestrogenized ♂♂ and ♀♀ (mildly) Intact ♂♂ and ♀♀ Castrate ♂♂ and ♀♀ Intact ♂♂+TP
157220 Line A	7–10	Oestrogenized ♂♂ Intact ♀♀	Castrate ♂♂ and ♀♀ Intact ♂♂+TP	Intact ♂♂
4061	2–7	Oestrogenized ♂♂	Intact ♂♂ and ♀♀	Castrate ♂♂ and ♀♀
4092	2–9	Oestrogenized ♂♂ Castrate ♂♂ and ♀♀	—	Intact ♂♂ and ♀♀ Intact ♂♂+TP
166189	7–9	Oestrogenized ♂♂ Intact ♀♀	Castrate ♀♀	Intact ♂♂ Castrate ♂♂ Intact ♂♂+TP
164041	4 and 5	Oestrogenized ♂♂ Intact ♀♀ Castrate ♂♂ and ♀♀	Intact ♂♂	—

TP = treatment with testosterone propionate . 5 mg twice weekly

These experiments were continued only until the animals in the most rapid growth groups had sizable tumours at which time all animals on the experiment were killed It is possible therefore that had the experiments been continued *longer growth* might have occurred in some animals in the inconsistent or no growth groups

as four months at which time the interstitial cells are still hypertrophied and show a moderate degree of hyperplasia

Although such treatment was ineffective in promoting growth of the completely dependent 157220 Line B tumour it did allow tumour growth in intact male mice bearing grafts of three lines of intermediate dependency (157220 Line A 166189 4658) However in two lines that grew only very inconsistently in castrate males (166189 4061) treatment of such animals with LH did not stimulate tumour growth Somewhat more surprising was the observation that when oestrogenized males bearing explants of either 157220 Line A or 166189 were treated with LH tumour growth was distinctly retarded Furthermore although grafts of either 157220 Line A or 4092 would grow in castrate female hosts the administration of LH to such recipients completely prevented growth It was most interesting to find that the administration of LH produced a tremendous interstitial cell hyperplasia in intact male mice bearing stilboestrol pellets at the same time that it was inhibiting the growth of 157220 Line A in the same mice

It seems most probable therefore that the administration of this LH preparation to intact male animals promoted tumour growth through its effect upon the hormone production by the testis for in the absence of testes treatment in most cases inhibited rather than enhanced tumour growth

Direct effect of oestrogen and influence of intrasplenic transplantation

It seemed possible that oestrogen might exert a direct stimulating effect on at least certain of these tumours In order to test this possibility experiments were set up in which bits of tumour were placed within the spleen and a small 10 per cent oestrone cholesterol pellet inserted next to the explant A second tumour graft was then placed in the subcutaneous tissues In this way it was hoped that the intrasplenic graft would reside in an environment of active oestrogen while the subcutaneous graft would not As a control cholesterol pellets were substituted for the oestrone pellets

tumour grow only in animals that are heavily oestrogenized (implanted with 10 per cent stilboestrol or oestrone pellets or *ingesting a diet containing 0 8 μg stilboestrol per gram)* and no other treatment has been found that is at all effective in promoting the growth of this tumour

Tumour 166189 has been of considerable interest for it appears to have increased in its dependency as transplantation has progressed As indicated in Table III by the seventh transfer it was growing as rapidly in intact females as in mildly oestrogenized males so that we began to carry" the tumour in intact female hosts Although the growth rate of the tumour in such hosts remained essentially constant over the next few transfer generations growth in mildly oestro genized castrate males became perceptibly slower By the 13th generation it required five months for tumours to grow to an *average weight of 382 mg in these oestrogenized males* while they grew to an average weight of 630 mg in intact females in only three months Explants to castrate female recipients ingesting the same stilboestrol containing diet as the males showed a rate of growth comparable to those in the intact female animals Furthermore in the following transfer generation only four of eleven mildly oestrogenized castrate male recipients grew tumours during an eight month period of observation These data suggest that this tumour gradually became more dependent on some factor present to a greater extent in female than in male mice Since this does not appear to be related to oestrogen stimulation, one wonders if it might not be of pituitary origin

Effect of administered LH

Because of our interest in the role of pituitary LH in the production of this type of tumour we have investigated the influence of administered LH upon their growth in trans plant Throughout these studies Armour s sheep pituitary LH No 227-80 was used all animals receiving 0 1 mg intra peritoneally each day On this regimen seminal vesicles of intact male mice remain well above normal size for as long

became evident Se\eral lmes that orgmally produced detectable amounts of androgen rather quickl) lost thıs abılity while three tumour lmes evolved that ha\e contmued to produce goodly amounts of androgen through as many as 22 serial transfers over a period of time exceeding fi\e years

The first studies carried out with these tumours were de sıgned to determıne ıf an ıncreased stımulatıon wıth pıtuıtary gonadotrophins would result in an increased production of androgenic hormones The tumours were allowed to grow to palpable size in castrate oestrogenized male recipients Approximately half of the animals were then injected daily with a horse pituitar) gonadotrophin preparation for a period of three weeks prior to being killed As will be noted in Table IV such treatment resulted in increased androgen production

Table IV

EFFECT OF GONADOTROPHIN THERAPY UPON ANDROGEN PRODUCTION BY TRANSPLANTS OF THREE INTERSTITIAL CELL TUMOURS INDUCED IN THE TESTES OF BALB/c MICE

Tumour No	*Treatment*	*No animals*	*Tumour size (av) (g)*	*Seminal vesicles wt (av) (mg)*	*Lt kidney wt (av) (mg)*
164041 3rd Trans	10% stılb pellet	9	0 77	207	281
	10° stılb pellet and gonadotrophın	9	0 73	484	391
164041 4th–6th	10% stılb pellet	16	1 77	104	217
Trans	10 ' stılb pellet and gonadotrophın	18	1 03	280	281
157°°0 Line A	0 2 µg stılb diet	8	0 80	56	165
14th Trans	0 2 µg stılb diet and gonadotrophın	7	0 65	175	193
157220 Line A	0 2 µg stılb diet	7	0 54	154	182
15th Trans	0 2 µg stılb diet and gonadotrophın	"	0 50	239	211
166189 8th Trans	0 2 µg stılb diet	8	0 46	89	168
	0 2 µg stılb diet and gonadotrophın	10	0 24	109	187

All treated mice received 0 1 mg of Armour s horse pituitary gonadotrophin No 316–115 intraperitoneally daily for 3 weeks prior to being killed

In the first few experiments, although microscopic bits of "active" tumour were usually found in the spleens near the oestrone pellets (but not in the subcutaneous tissues) they were frequently noted at some distance from the pellets and also not infrequently about the cholesterol pellets An experiment was therefore set up in which the completely dependent 157220 Line B tumour was implanted both intrasplenically and subcutaneously into 11 ovariectomized female animals that were then placed on the diet containing 0 2 μg stilboestrol per gram After five months nine of these animals were found to have macroscopic tumour growths within the spleen but none showed evidence of growth of the sub cutaneous explants This and other experiments suggest that the intra abdominal position definitely favours tumour growth

However experiments with two different tumours also suggest that oestrogen does exert a direct stimulating effect In one using an early transfer generation of 4658, nine of 11 intact male mice bearing intrasplenic oestrone pellets showed areas of active' tumour next to the pellet while no such areas were seen in the spleens of six intact males bearing cholesterol pellets Again employing intact male mice and 157220 Line B, six of 11 animals had small areas of tumour immediately adjacent to an oestrone pellet while no areas of tumour were noted in 12 animals with cholesterol pellets

Hormone Production

A study of the hormones produced by these transplantable interstitial cell tumours has been most interesting Initially it was evident that many of the transplants were producing appreciable amounts of androgenic hormones as evidenced by the presence of secretion in the seminal vesicles of castrated oestrogenized male recipients Early in the course of trans plantation considerable variability was noted in the amounts of androgen being produced by different explants from the same donor tumour, but with serial transplantation more uniformity of function within the various lines of tumour

indicate that tumour line 4658 releases into the circulation significant amounts of oestrogenically active hormones but little or no progestational hormones 4061 releases significant amounts of progestational hormones but no detectable amounts of oestrogens while 4092 apparently secretes bio logically detectable amounts of both Similar studies have been carried out employing the three androgen producing tumours but in these it was impossible to determine histo logically if oestrogenic and/or progestational hormones were being produced in addition to androgens

It is of considerable interest to note that no absolute correlation existed between the production of hormones by these tumours and either the degree of dependency or the rate of growth exhibited by them To be sure during the first few passages several of the tumour lines did tend to increase their rate of growth to lose some of their dependency and to decrease or lose their ability to produce androgens Tumour 164041 on the other hand after 22 serial passages still produces as large quantities of androgen as it did two years ago when in its eighth transfer generation During this time its growth rate essentially tripled and it became less dependent now growing as rapidly in intact male recipients as it does in castrate oestrogenized ones In contrast tumour 157220 Line B has over a four year period remained completely dependent growing subcutaneously only in animals that are heavily oestrogenized and requiring about five months to attain an appreciable size This tumour in spite of its slow growth rate and high degree of dependency has never produced histologically detectable amounts of androgenic hormones even when under the influence of excessive stimula tion with exogenous gonadotrophins

REFERENCES

Andervont H B Shimkin M B and Canter H Y (1957) *J nat Cancer Inst* 18 1
Ely C A (1953) *Proc Soc exp Biol* N Y 84 501
Gardner W U (1948) *Cancer Res* 8 397

by all three of these tumour lines A fourth non androgen producing tumour line was studied in a similar fashion but in this case additional stimulation with gonadotrophin did not result in the production of histologically detectable amounts of androgen

As our studies of hormone biosynthesis progressed it became important to determine if the non androgen producing tumour lines were secreting biologically detectable amounts of other types of hormones Three such lines were trans planted to castrate female recipients who were then placed on the semisynthetic diet containing 0 2 µg stil boestrol per gram This amount of stilboestrol inhibits the development of hormone producing adrenal hyperplasia and allows these particular testicular tumours to grow readily When the tumours became palpable the animals were placed on a non oestrogen containing diet for a period of two weeks prior to being killed The submaxillary salivary glands uteri and vaginae were then examined microscopically The results are summarized in Table V These studies

Table V

HISTOLOGICAL EVALUATION OF HORMONE PRODUCTION BY TRANSPLANTS OF THREE NON ANDROGEN PRODUCING INTERSTITIAL CELL TESTICULAR TUMOURS INDUCED IN BALB/c MICE

Tumour	*Number of animals*	*Submaxillary Gland*	*Uterus*	*Vagina*
4058	6	Female type	90 0 mg Av Wt Little tendency for stromal loosening	Partially corni fied epithelium
4061	13	Female type	91 0 mg Av Wt Extensive progesta tional changes	Unstimulated epithelium
4092	21	Female type	98 5 mg Av Wt Marked progesta tional changes	Mucified epithelium
Without tumour	12	Female type	27 8 mg Av Wt Unstimulated	Unstimulated epithelium

The ovariectomized female recipients were fed a diet containing 0 2 µg stilboestrol per gram until 2 weeks prior to being killed to prevent the development of sex steroid producing adrenal hyperplasias

STEROID BIOSYNTHESIS IN INDUCED TESTICULAR INTERSTITIAL CELL TUMOURS OF MICE

OSCAR V DOMINGUEZ LEO T SAMUELS AND ROBERT A HUSEBY

Department of Biological Chemistry University of Utah College of Medicine Salt Lake City and Department of Surgery University of Colorado School of Medicine Denver

INDUCED interstitial cell tumours of the mouse testis that continue to produce hormones after transplantation afford excellent material with which to study the biosynthesis of steroid hormones in neoplastic tissue All studies of the genesis of these tumours have indicated that they arise from once normal interstitial cells or their precursors thus apparently eliminating the possibility ever present in the case of many spontaneously occurring testicular tumours that the neo plastic cells arose either from adrenal rest cells or as a unilateral teratoma from totipotent germinal cells Further more the serial transplantation of these tumours results in the development of essentially pure *in vivo* cultures of abnormal interstitial cells free from other testicular elements

From the studies of a number of workers (Hechter *et al* 1951, Slaunwhite and Samuels 1956 Hayano *et al* 1956 Meyer 1955*a* *b*) a system of biosynthesis of the various steroid hormones from pregnenolone can be outlined (Fig 1) Accord ing to this plan the separation of the C_{19} and C_{18} steroids from the C_{21} hormones occurs at 17α hydroxyprogesterone By choosing different steroid substrates and co factors the relative activities of the different enzyme systems in different tissues can be compared

In the studies discussed here the conversion of 5 pregnen 3β ol 20 one to progesterone in the presence of excess di phosphopyridine nucleotide (DPN) was used as a measure of

HUSEBY R A (1954) *Proc Amer Ass Cancer Res* 1 No 2 21
HUSEBY R A and BITTNER J J (1952) *Cancer Res* 12 271
JULL J W (1954) *Brit J Cancer* 8, 704
SMELSER G K (1933) *Physiol Zoöl* 6, 396

[This paper and the following one were presented by Dr Huseby as a single communication and discussion was postponed until after Prof Gardner s second paper (see p 249) —EDS]

3β ol dehydrogenase activity The conversion of [4 [14]C]progesterone to the various steroids in the presence of DPN adenosine triphosphate (ATP) and fumarate gave a relative measure of a number of the subsequent steroid transformations since the radioactivity could only be lost if ring A of the steroid was ruptured this does not seem to occur in significant amounts under *in vitro* conditions In estimating relative activities where a series of reactions occurs however the activity of any step must be considered in relation to the relative availability of the particular substrate involved and the rate at which the product is removed [21 [14]C]17α Hydroxyprogesterone was used as substrate in the presence of DPN ATP and fumarate to measure the $C_{(17)}$ side chain splitting activity Since previous findings in this laboratory indicate that this reaction is essentially irreversible and that there is no significant product inhibition the extent to which the radioactive carbon was present as a water soluble volatile acid was a measure of the $C_{(1\ )}$ side chain splitting enzyme system The extent to which 17α hydroxyprogesterone was converted into specific more polar radioactive steroids correcting for

---

FIG 1

I Δ^5 Pregnen 3β ol 20-one (Pregnenolone)
II Progesterone
III 17α Hydroxyprogesterone
IV Cortexone
V 17α Hydroxycortexone (Reichstein s Compound S)
VI Aldosterone
VII Corticosterone
VIII 17α Hydrocortisone (Kendall s Compound F) (Cortisol)
IX Cortisone (Kendall s Compound E)
X Δ^4 Androstene 3 17 dione (Androstenedione)
XI Testosterone
XII Oestradiol
XIII Oestrone
XIV Oestriol
XV 17α Hydroxypregnenolone
XVI Dehydro*iso*androsterone
XVII 11β Hydroxyandrostenedione
XVIII 6β Hydroxyprogesterone
XIX 19 Hydroxy Δ androstene 3 17-dione
XX 18 Aldo-cortexone
XXI *Adrenosterone* (Δ^4 androstene 3 11 17 trione)
XXII 6μ Hydroxycortisol

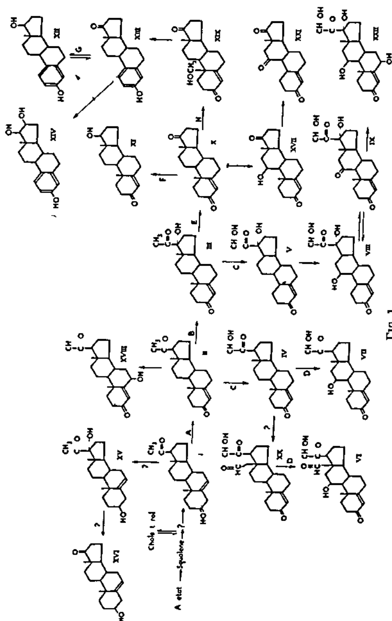


Fig 1

out in the presence of non radioactive carrier and the chromatographic behaviour determined thereafter Finally oxidation or reduction and subsequent chromatography were used to establish the character of certain steroids

The more pertinent findings to date have been summarized briefly in Table I It is immediately evident that the relative concentrations of the various enzymes necessary for conversion of pregnenolone to 4 androstenedione differ considerably from tumour to tumour and in all instances are different from that seen in the normal testis Of course any direct comparison of the enzyme content of normal and neoplastic interstitial cells is impossible since in the normal testis interstitial cells constitute but a very small fraction of the total tissue mass The possibility should also be entertained that the tubular tissues of the testes might under the conditions of the incubation metabolize to some extent either the substrates added or the compounds formed from them by the interstitial cells

As reported previously there appears to be no correlation between the production of androgens by these tumours and their concentration of steroid 3β ol dehydrogenase (Huseby Samuels amd Helmreich 1954) Both of the non androgen producing tumours so far studied appear however to be deficient in 17 hydroxylase In spite of this tumour 4658 produces detectable amounts of oestrogenically active substances *in vivo* and *in vitro* converts progesterone to phenolic compounds (as yet unidentified) Furthermore the formation of phenolic compounds apparently occurs as readily when progesterone is incubated with this tumour as it does when testosterone is used as the substrate The exact significance of these observations relative to the role of 17 hydroxylase in oestrogen biosynthesis must await the results of further investigations

It was of considerable interest to find that with the other non androgen producing tumour, 4061 a large amount of the progesterone substrate was converted to more polar neutral compounds although neither 17α hydroxyprogesterone nor 4 androstenedione was identifiable at the end of the incubation Although characterization of all the principal compounds

the loss of substrate by side chain splitting was a measure of the $C_{(21)}$, $C_{(11)}$ and possibly $C_{(6)}$ hydroxylases

Homogenates were prepared from non necrotic portions of the tumours dissected free of surrounding normal tissue after any bloody cysts had been opened and drained The weighed tissue was homogenized in a known volume of a mixture of equal parts of Krebs bicarbonate buffer pH 7 4 and bovine blood serum containing 0 04 M nicotinamide A Ten Broek homogenizer which was kept cold in crushed ice was used for the homogenization Amounts of homogenate equivalent to the desired amount of tissue (100 mg in the case of all sub strates except pregnenolone 15 mg in the case of the latter) were pipetted into 125 ml glass stoppered Erlenmeyer flasks, enough of the serum buffer nicotinamide mixture containing the co factors was added to bring the total volume to 20 ml and the incubation was carried out at 37° for 180 min in an atmosphere of 95 per cent O_2 5 per cent CO_2

Enzyme activity was stopped by adding enough KOH to make the pH of the solution slightly greater than 10 and shaking the mixture with an equal volume of ethyl ether Ether was used because of the limited solubility of nicotin amide in this solvent The extraction with ether was repeated five times and the extract washback washed with water to neutrality dried over sodium sulphate and the solvent driven off under nitrogen This fraction contained the neutral steroids

Those flasks with substrates which contained ^{14}C at position 4 were then acidified and the aqueous layer was extracted with a mixture of equal parts of hexane and ethyl acetate This extract was washed to neutrality water was removed with sodium sulphate and the organic phase was dried down under nitrogen as before The fraction was considered to represent the phenolic steroids

The lipid fractions were chromatographed in various form amide systems as described by Zaffaroni (1953) When the positions of radioactive spots indicated possible alcoholic steroids acetylation of the radioactive compounds was carried

formed in this incubation has not been completed it has been established that about 18 per cent of the progesterone was converted to cortexone Similarly, in the incubations of this tumour with 17α hydroxyprogesterone identifiable amounts of Reichstein s compound S were recovered Thus it is evident that this tumour of testicular interstitial cell origin contains 21 hydroxylase an enzyme more usually associated with adrenal cortical tissue

Considering the androgen producing tumours the mildly masculinizing 157220 Line A was found to contain significantly less $C_{(17)}$ side chain splitting activity and also probably less 17 hydroxylase activity than does the very masculinizing 164041 It is therefore possible that one or both of these enzyme steps could be rate limiting as far as androgen production by this tumour is concerned Such is certainly not the case however in tumour 166189 Although this is only moderately masculinizing as compared to 164041 it apparently contains appreciably greater amounts of all three enzymes involved in the conversion of pregnenolene to 4 androstene dione suggesting that the rate limiting step in the production of androgens occurs prior to the formation of pregnenolone This is presently under investigation employing both acetate and cholesterol as substrates

Before a complete picture of steroid biosynthesis in these induced interstitial cell tumours can be obtained a good deal more work will obviously be required Not only will all the major compounds formed in these incubations have to be identified but also the pathways leading to the formation of pregnenolone and the enzymic changes that occur in interstitial cells prior to the development of frank neoplasms will have to be investigated From the data on hand however it would appear that during the process of tumorigenesis and serial transplantation the loss of steroid biosynthetic enzymes occurs in a more or less random fashion and that this loss need not of necessity reflect either the growth rate or the degree of dependency of the resultant tumour It will of course be of importance also to ascertain if some enzymes such as

Table I

COMPARISON OF HORMONE PRODUCTION AND ACTIVITY OF THREE BIOSYNTHETIC ENZYME SYSTEMS IN NORMAL TESTES AND IN FIVE TRANSPLANTED INTERSTITIAL CELL TESTICULAR TUMOURS INDUCED IN BALB/c MICE

Tissue	*Hormone Production*	*5 Pregnen 3β ol 20 one incubated with 15 mg of tissue+DPN*	*[4 ^{14}C]Progesterone incubated with 100 mg tissue+DPN+ATP+fumarate*	*[21 ^{14}C]17α hydroxyprogesterone incubated with 100 mg tissue +DPN+ATP+fumarate*
Normal Testes	Androgenic (SV 186 mg)	- - - - - *	58% altered 5% as 4 androstenedione 11% as 17α hydroxyprogesterone 10% as phenolic comps	3ر% removal of $C_{(17)}$ side chain
Tumour 104041	Androgenic (SV 363 mg)	23% as progesterone	65% altered 37% as 4 androstenedione 9% as 17α hydroxyprogesterone 8% as phenolic comps	60% removal of $C_{(17)}$ side chain
Tumour 166180	Androgenic (SV 186 mg)	40% as progesterone 3% as 4 androstenedione	90% altered 74% as 4 androstenedione 0% as 17α hydroxyprogesterone 0% as phenolic comps	80% removal of $C_{(17)}$ side chain
Tumour 1ر72°0 Line \	Androgenic (SV 93 mg)	30% as progesterone	46% altered 12% as 4 androstenedione 10% as 17α hydroxyprogesterone 8% as phenolic comps	16% removal of $C_{(17)}$ side chain
Tumour 46ر8	Non androgenic Oestrogenic ? Progestational	27% as progesterone	17% altered 0% as 4 androstenedione 0% as 17α hydroxyprogesterone 6% as phenolic comps	13% removal of $C_{(17)}$ side chain
Tumour 4061	Non androgenic Non oestrogenic Progestational	46% as progesterone	47% altered 0% as 4 androstenedione 0% as 17α hydroxyprogesterone 0% as phenolic comps	20% removal of $C_{(17)}$ side chain

Due to the production of androgens by the first three tumours histological detection of progestational or oestrogenic hormones was impossible All compounds formed have not as yet been identified 0% indicates that radioactivity was not significantly above background and in these experiments means that less than 2% of the substrate had been converted to the compound in question

(DPN = diphosphopyridine nucleotide ATP = adenosine triphosphate)

* Normal testes contain 3β-ol dehydrogenase but this has not yet been quantified by the new method in which progesterone is actually identified

TESTICULAR TUMORIGENESIS*

W U GARDNER
Yale University School of Medicine

TWENTY years ago Harold Burrows (1937) in London wrote a little two page note entitled, Acquired resistance to oestrone in a male mouse He described the re establishment of secretory activity in the seminal vesicles and prostate of an oestrogen treated mouse that had a nodular growth of testi cular interstitial tissue This mouse had the first hormonally induced testicular interstitial cell tumour (ICT) to be described although the author himself did not so designate it at the time Burrows (1935) and Gardner (1937) had described hyper trophy and hyperplasia of the interstitial tissue of the testes of oestrogen treated mice The latter investigator had noted that this change occurred almost exclusively in the testes of mice of the A strain Burrows experiences had been with stock mice Three years later Bonser and Robson (1940) and Hooker Pfeiffer and Gardner (1940) reported that ICT appeared in mice given triphenylethylene stilboestrol (3 4 di p hydroxyphenylhex 3 ene) and oestradiol benzoate Mice of the RIII and CBA strains did not acquire ICT when similarly treated

Subsequently the C strain (Shimkin Grady and Andervont 1941) and JK strain (Gardner 1943*a*) were added to the list of mice susceptible to ICT following oestrogen treatment Mice of the C strain acquired tumours after pellets of stilboestrol had been removed after having been retained for from 4 to 16 weeks (Andervont Shimkin and Canter 1957) Mice of this strain were usually susceptible to ICT

The original observations reported in this paper have been supported in part by grants from the Anna Fuller Fund the Jane Coffin Childs Memorial Fund and by Grant C-343 National Cancer Institute U S Public Health Service

21 hydroxylase, can arise or at least increase significantly in concentration during tumorigenesis

REFERENCES

HAYANO M SABA N DORFMAN R I and HECHTER O (1956) *Recent Progr Hormone Res* 12 79

HECHTER O ZAFFARONI A JACOBSEN R P LEVY H JEANLOZ R W SCHENKER V and PINCUS G (1951) *Recent Progr Hormone Res* 6 215

HUSEBY R A SAMUELS L T and HELMREICH M L (1954) *Proc Soc exp Biol N Y* 86 580

MEYER A S (1955*a*) *Experientia* 11 99

MEYER A S (1955*b*) *Biochim biophys Acta* 17 441

SLAUNWHITE W R and SAMUELS L T (1956) *J biol Chem* 220 341

ZAFFARONI A (1953) *Recent Progr Hormone Res* 8 51

[Discussion after this paper and the preceding one was postponed until after the second paper by Prof Gardner (see p 249) —EDS]

and of the non susceptible C3H strain were transplanted sub cutaneously into gonadectomized hybrid hosts ICT appeared in the grafted testes of strain A donors in the oestrogen treated hybrids and in one of the grafted testes from C3H donors (Table I) One testis from a C3H donor had a very small

Table I

TUMOURS IN TRANSPLANTED TESTES IN MALE MICE GIVEN STILBOESTROL-CHOLESTEROL (1 PART TO 3 PARTS) TESTES OF NEWBORN MICE WERE TRANSPLANTED IN HOSTS 29 TO 108 DAYS OF AGE (TRENTIN AND GARDNER 1957)

Donor	*Host*	*Number*	*Number of tumours*
A	A ♀	9	3
C3H	C3H ♀	12	1 nodule
A	(A × C3H) ♀	42	26
C3H	(A × C3H) ♀	49	1
(A × C3H)	(A × C3H) ♀	14	4

nodule of interstitial cells after being grafted into a C3H host These experiments reaffirmed the strain specificity of the pre disposition for ICT and indicated that this predisposition was located in the testis itself Furthermore they demonstrated that the normal blood supply and innervation were not essen tial The most direct approach for further investigation should be provided by studies of testes rather than of the environ ment in which the ICT form (Tables I and II)

Table II

TUMOURS IN THE INTACT TESTES OF A C3H AND F_1 HYBRID MICE BEARING SUBCUTANEOUS IMPLANTS OF PELLETS OF STILBOESTROL-CHOLESTEROL CONTAINING 25 STILBOESTROL (TRENTIN AND GARDNER 1957)

Strain	*Number treated*	*No of tumours*	*Weight of tumours average mg*
A	12	5	480
C3H	16	0	—
A × C3H	15	7	568

Castrated rats bearing intrasplenic grafts of testes for pro longed periods acquired testicular tumours some of which were composed of interstitial cells (Biskind and Biskind 1945 Twombly Meisel and Stout 1949)

Spontaneous ICT ICT appeared in mice of one inbred strain without oestrogen treatment (Athias, 1945) Male mice of this strain interestingly also frequently had mammary tumours Three ICT appeared in hybrid mice serving as controls for other experiments (Gardner, 1943*b*) These mice were from hybrid groups susceptible to such tumours when oestrogen was injected One ICT that appeared in an untreated mouse of the A strain showed an unusual pleomorphism (Hooker Strong and Pfeiffer 1946) and a capacity to undergo histological and functional changes subsequent to treatment with gonadotrophins

Causal genesis The hypertrophy of the interstitial cells was assumed to be a result of the action of oestrogen on the pituitary which resulted in an increase in luteinizing hormone LH (Gardner 1937) A similar hypothesis had been formulated to explain the cyclic occurrence of oestrus in normal female rats (Lane 1934) The injection of pregnant mare's serum gonadotrophin for prolonged periods resulted in changes comparable to early stages of interstitial cell tumour formation in mice of the A strain (Pfeiffer and Hooker 1943) Attempts to assay pituitary glands of oestrogen treated mice in hypophysectomized animals failed to reveal detectable amounts of gonadotrophin (Gardner, unpublished) or differences between those strains susceptible to or resistant to ICT The intratesticular implantation of small pellets of oestrogens did not result in ICT, indicating that a direct action of the oestrogen was improbable A number of observations, however indicated that the pituitary was essential but that the testes of the susceptible animals had some unique characteristics Hypophysectomized mice have not survived long enough to provide convincing evidence of the influence of the pituitary on formation of ICT The injection of anti hormone does indicate that pituitary gonadotrophin is essential but the experiments are not conclusive (Ely 1953)

Convincing evidence now exists that the specific quality favouring ICT formation is located in the testis (Trentin and Gardner, 1957) Testes of mice of the ICT susceptible A strain

studied Mice of the A strain that have received oestrone oestradiol benzoate oestradiol equilene benzoate stilboestrol and triphenylethylene have acquired testicular tumours (Bonser and Robson 1940 Hooker and Pfeiffer 1942 Gardner 1943*a* Gardner unpublished)

A number of years ago we noted that hybrid (C57 × CBA) mice given tri *p* anisyl chloroethylene (TACE) had ICT al

Table III

INFLUENCE OF TRIPHENYLETHYLENE (TPE) AND TRI *p* ANISYL CHLOROETHYLENE (TACE) ON THE APPEARANCE OF INTERSTITIAL CELL TUMOURS IN MICE OF DIFFERENT INBRED STRAINS OR HYBRID GROUPS (DATA FROM GARDNER 1943*a* GARDNER AND BODDAERT 1950 GARDNER 1957)

Oestrogenic chemical	*Strain*	*No of animals reported*	*No of ICT*	*Duration of treatment Months*	*No of pituitary tumours*
TPE	JK*	13	7	8–16	
	A	17	7	15–18	
	C3H*	6	1	9–22	
	C12I*	5	0	18–25	
	N*	[illegible]	0	13–21	
TACE	CBA	17	0	12–22	0
	A	15	0	10–1[illegible]	0
	C57*	27	8	11–19	0
	C3H*	14	0	12–18	0
	BC*	37	0	10–21	5
	BC × C57*	27	0	10–27	17
	A × C57	14	10	15–23	0
	A × C3H	24	4	11–32	0
	C57 × CBA*	92	46	8–[illegible]8	5

* Mice of these strains have not had ICT after injections of stilboestrol or oestradiol benzoate Mice of the strains or group not so designated have ICT when given stilboestrol or oestradiol benzoate (Gardner 1957)

though similar hybrids did not have such tumours when oestradiol or its esters or stilboestrol was injected (Gardner and Boddaert 1950 Gardner 1941) Mice of the A CBA C3H or BC strains or hybrids (BC × C57) did not acquire ICT when given TACE (Table III) Mice of the C57 strain and A × C57 hybrids had ICT when given TACE Four of 24 A × C3H hybrids had ICT but such tumours also appeared in similar mice given stilboestrol or oestradiol benzoate (Gardner 1957,

Histogenesis of ICT The sequences of changes in the testes of oestrogen treated mice of the strains susceptible to ICT were described in considerable detail by Hooker and Pfeiffer (1942) After oestrogen treatment was initiated the interstitial cells first hypertrophied and then degenerated usually with the appearance of ceroid Subsequently the glandular interstitial cells reappeared, apparently by origin from fibroblast like cells Focal areas of hypertrophy and hyperplasia resulted in the formation of small nodules of glandular interstitial cells Small areas of hyperchromatic and particularly hyperplastic cells were presumably the major sources of new cells Some of these small cells enlarged to resemble normal functional interstitial cells Mitotic figures were most common in the small glandular cells (second generation) or the small hyperchromatic cells (third generation)

The testes of mice of the non susceptible strains reacted similarly through the stage of degeneration Replacement of the interstitial cells did not occur in these strains however and the testes and genital tissues continued to regress unless oestrogens were withheld (Gardner, 1937)

Hormonal specificity The statement has been made that oestrogens differ in carcinogenic activity in direct proportions to their different oestrogenic activities Because oestrogenic activity is usually determined by the response of the vaginal mucosa this implies a correlation between vaginal activity and carcinogenic activity The difficulties of comparing different oestrogens of such contrasting activities as triphenylethylene and oestradiol or their derivatives or esters prevent too much emphasis on this generalization Triphenylethylene was classed as a pro oestrogen (Emmens, 1942) because it was but little more effective when applied directly to the vagina than when applied subcutaneously at areas remote to the vagina The oestrogenic oestrone derivatives that have been tested stilboestrol and triphenylchloroethylene, are all oestrogens with a *high systemic local response ratio* (Emmens 1942) Triphenylethylene seems to be more effective in inciting the formation of ICT than the other oestrogens that have been

The possibility that susceptibility to ICT among C57 mice is different from that for A mice must be considered Hybrid mice (A × C57) acquired both pituitary tumours and ICT when given stilboestrol or oestradiol but only ICT when given TACE The high incidence of ICT in these mice might indicate an additive influence of genetic susceptibilities The experiment undertaken by Trentin and Gardner (1957) might well be repeated with the BC and C57 strains

Transplantation of ICT The number of ICT that grow subsequent to transplantation into related mice cannot be ascertained too well from the published reports because failures may not be mentioned Three tumours considered to be malignant because they had metastasized failed to grow upon transplantation (Bonser 1942) One ICT arising in an A strain mouse grew through four generations of transplantation but only in oestrogen treated mice (Gardner, 1943*a*) The tumour continued to grow after it had become established when oestrogen injections were discontinued and in one experiment growth of established transplanted tumours continued after hypophysectomy Further studies showed other tumours to be dependent for growth subsequent to transplantation upon the presence of exogenous oestrogens The grafts could remain dormant for periods of over six months and then grow subsequent to oestrogen treatment (Gardner 1945) Usually the first transfer appeared after some delay and grew slowly later growth occurred more rapidly Not all ICT were dependent upon oestrogens for growth subsequent to transplantation (Jull 1954) especially after transplantation for several generations Andervont Shimkin and Canter (1957) transplanted 70 tumours that arose in oestrogen treated BALB mice and 56 of them grew Twenty one were independent of oestrogen in the first transfer generation These ICT continued to grow in the original donor subsequent to removal of the stilboestrol pellets or when they had appeared after the pellets of stilboestrol had been removed Six tumours that arose in mice carrying stilboestrol pellets grew independently of oestrogen treatment after the fourth transfer generation

and Table III) Whether TACE is a pro oestrogen or not, is not known but it seems to be usually effective in inciting ICT Eight of 14 C57 mice given tri p anisyl iodoethylene also had ICT after 512 to 756 days of treatment (Gardner unpublished)

Mice of the C57 strain (Gardner and Strong 1940) and their hybrids (C57 × C3H Gardner, 1954, and C57 × CBA, Gardner, 1941) acquire pituitary tumours after the injection of oestradiol esters or stilboestrol (Table IV) Pituitary tumours

Table IV

APPEARANCE OF ICT IN HYBRID MICE GIVEN OESTRADIOL OESTRONE OR THEIR ESTERS AND STILBOESTROL OVER PROLONGED PERIODS (DATA FROM GARDNER 1957)

	Treatment					
	Oestradiol etc			*Stilboestrol*		
Strain or Group	*No of mice*	*No of tumours*	*Average age at tumour*	*No of mice*	*No of tumours*	*Average age at tumour*
A × C57	47	1	350	10	1	468
C57 × A	53	6	495	12	3	411
A × C3H	55	6	486	14	2	434
C3H × A	70	7	413	11	4	390
C57 × CBA	43	—	(431)*	10	—	(561)*
CBA × C57	38	—	(427)*	10	—	(4 3)*

* The average age of survival of all the mice in the group

appeared in about 80 per cent of the male hybrids (CBA × C57) that received these oestrogens (Gardner, 1941) but in only about 5 per cent of these hybrids that received TACE (Gardner and Boddaert 1950) None of the mice of the C57 strain given TACE had pituitary tumours On the other hand five of the TACE treated BC mice and 17 of the TACE treated hybrids (C57 × BC) had pituitary tumours but none had testicular tumours (Gardner unpublished) This indicates an inverse relationship between pituitary tumours and ICT (Table III) Mice of the A C3H and CBA strains however, acquired neither ICT nor pituitary tumours when given TACE

A hormonal modification seems to be required for the initiation of the ICT and many tumours require oestrogen for the subsequent proliferation of cells as well The duration of the modified hormonal environment required for the initiation of ICT may be quite short in BALB mice (Andervont Shimkin and Canter 1957) The tumour cells however, may survive prolonged periods in quiescence and grow after a proper environment has been attained Tumours all seem to become independent of oestrogen treatment after continued transplantation The writer has had four ICT that became independent by the eighth transfer generation and one that had attained independence by the fifth generation If larger numbers of hosts were used it is not improbable that more tumours would show hormonal independence at earlier periods and that a number of variants might arise from a single tumour Convincing evidence that the progressive changes towards autonomy are acquired simultaneously by all cells of the tumours has not been obtained The general habit of transplanting from the first tumour to attain a reasonable size in each generation may tend to select for greater hormonal independence

Hormone production by ICT Androgenic effects are produced by ICT as was first indicated by Burrows' (1937) observation Comparable observations have been made repeatedly by most investigators The seminal vesicles of the oestrogen treated mice which acquire ICT that attain considerable size often become filled with secretion and the prostate is restored Animals bearing transplanted tumours for at least five generations have shown evidence of hormone production by dependent tumours (Gardner 1943, Bjorn and Gardner 1956) Female tumour bearing hosts acquire large inguinal hernias and enlarged clitorises (Figs 1 and 2)

Androgen production has occurred in mice with tumours that enlarged the testis and were composed of cells classed as type 1, type 2 or mixtures of types 1 and 2 or of types 2 and 3 No tumours composed entirely of the small hyperchromatic cells classed as type 3 were observed More than half of the



FIG. 1. On the left is ... m l m ... e 8 days after a second transfer generation of an ICT ... had been transplanted subcutaneously. The tumour measured 14 ... 3 mm ... Stilboestrol had been injected weekly at the rate of 20 μg into the ... of ... The scrotal testes were approximately one half normal size and yellow ... They contained little normal interstitial tissue. The seminal vesicles were empty but larger than in castrated animals. On the right hand side the photograph shows a female mouse in the fifth transfer generation that had a subcutaneous graft that measured 1 ... × 8 mm. This mouse showed large bilateral inguinal hernias and an enlarged clitoris. This mouse had received a pellet containing stilboestrol and cholesterol at the time the transplant was made. The uterus showed evidence of oestrogen and androgen. The ... mm interpubic ligament also indicated that the tumour was producing something that inhibited the osseous effects of oestrogen (Bjorn and Gardner 1956)

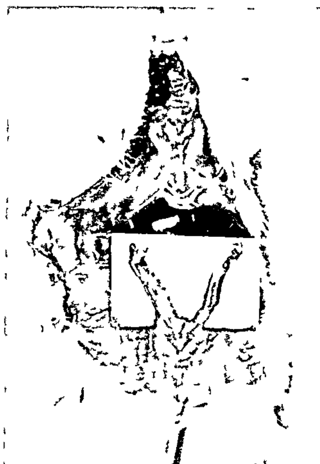


Fig 2 Same mouse as shown on right side in Fig 1 The exposed inguinal hernias contained gut and mesometrial fat The uterus is large This tumour (ATI 10 Gardner 1943*a*) first grew in mice not given oestrogen in the eighth transfer generation and it grew again in the twelfth generation In neither instance did it grow in all of the mice It was not transplanted from the mice with the tumours that grew without oestrogen

animals that had tumours that did not enlarge the testes also showed evidence of androgen production (Gardner and Boddaert 1950) particularly when cells of type 2 were present (six of eight animals)

Critical attempts to quantify hormone production by these tumours have not been made but certainly it is less than in normal interstitial tissue and is not apparently regulated in the same manner Oestrogen inhibits hormone production by normal interstitial tissue presumably by reducing the production of gonadotrophin It does not inhibit the production of androgen by the tumours Whether or not hormone production may depend upon the presence of pituitary gonadotrophins has not been investigated sufficiently It seems that some tumours may require gonadotrophin but that others may not

The possibility must be considered that those ICT that grow without oestrogen have acquired the capacity to produce their own requirements of hormone of a suitable type rather than a capacity to grow without the oestrogenic hormone

The uteri of some mice bearing transplanted ICT are well developed indicating the production of some oestrogen and the mammary glands of some males have grown (Gardner unpublished) It is however difficult to prove that the tumours are really producing oestrogen oestrogen may be a conversion product In our laboratory ICT have only been carried for up to 47 transfer generations and the tumour seemed functionally active at that time

Summary

Testicular interstitial cell tumours have appeared in oestrogen treated mice of some strains but not in others The tumours seem to depend upon a definite modification of the hormonal balances of the hosts for their initiation This may be an increase in the amount of luteinizing hormone but this has not been proven The subsequent growth of many ICT also depends upon a specific hormone adjustment conditioned by

oestrogen Most, if not all, tumours acquire an independence of oestrogen, sometimes by the time they attained transplant able sizes but in other instances only after several transplant generations The interval between initiation and attainment of autonomy may differ greatly in mice of different strains

The intrinsic capacity to become tumours seems to be located in the testis rather than in the other hormone pro ducing or destroying organs of the mice

Some of the triphenylethylene derivatives seem unusually effective in inducing ICT in strains of mice not generally susceptible to them when other oestrogens are given

The tumours produce androgenic effects in the host and occasionally oestrogenic effects They usually have to attain considerable size before the hormonal effects become apparent

REFERENCES

ANDERVONT H B SHIMKIN M B and CANTER H Y (1957) *J nat Cancer Inst* 18 1
ATHIAS M (1945) *Arch Pat Lisbon* 17 397
BISKIND M S and BISKIND G R (1945) *Proc Soc exp Biol N Y* 59 4
BJORN J and GARDNER W U (1956) *Endocrinology* 59 48
BONSER G M and ROBSON J M (1940) *J Path Bact* 51 9
BONSER G M (1942) *J Path Bact* 54 149
BURROWS H (1935) *J Path Bact* 41 218
BURROWS H (1937) *J Path Bact* 44 699
LLY C A (1953) *Proc Soc exp Biol N Y* 84 501
EMMENS C W (1942) *J Endocrin* 3 168
GARDNER W U (1937) *Anat Rec* 68 339
GARDNER W U (1941) *Cancer Res* 1 345
GARDNER W U (1943a) *Cancer Res* 3 92
GARDNER W U (1943b) *Cancer Res* 3 757
GARDNER W U (1945) *Cancer Res* 5 497
GARDNER W U (1954) *J nat Cancer Inst* 15 693
GARDNER W U (1957) Proc 2nd Canad Cancer Conf p 207 New York Academic Press Inc
GARDNER W U and BODDAERT J (1950) *Arch Path (Lab Med)* 50 750
GARDNER W U and STRONG L C (1940) *Yale J Biol Med* 12 543
HOOKER C W PFEIFFER C A and GARDNER W U (1940) *J Amer med Ass* 115 443
HOOKER C W and PFEIFFER C A (1942) *Cancer Res* 2 759

HOOKER C W STRONG L C and PFEIFFER C A (1946) *Cancer Res* 6 503
JULL J W (1954) *Brit J Cancer* 8 704
LANE C E (1934) *Amer J Physiol* 116 681
PFEIFFER C A and HOOKER C W (1943) *Cancer Res* 3 762
SHIMKIN M B GRADY H G and ANDERVONT H B (1941) *J nat Cancer Inst* 2 65
TRENTIN J J and GARDNER W U (1957) *Cancer Res* in press
TWOMBLY G H MEISEL D and STOUT A P (1949) *Cancer* 2 884

DISCUSSION

Woolley Prof Gardner did you use doisynolic acid as an oestrogen in your series?

Gardner No nor did we use any oestrogen with the modified D ring

Horning Dr Huseby did you observe any proliferation of mammary gland epithelium in your cryptorchid experiments?

Huseby In mice that have been made cryptorchid only we do not see any appreciable mammary gland development However since such animals have a good production of androgen by their testes I do not believe that the lack of mammary gland proliferation rules out the possibility that such testes are also producing a fair amount of oestrogen

Horning It has been shown recently in experiments on the rat that proliferation of the Leydig cells is sufficient to induce proliferation of the mammary gland as it has been shown that the interstitial cells are capable of secreting oestrogens as well as androgen

Have you observed the effect of hypophysectomy on the induction of interstitial cell tumours?

Huseby No we are just now beginning to set up experiments with hypophysectomy

Dorfman Dr Huseby has dropped two small bombshells One of them is that he tells us that pure testicular tissue can perform 21 hydroxylation a function which we believed to be highly specific for adrenal tissue In a human testicular tumour where we observed a similar phenomenon there was some question whether we were actually dealing with a testicular tumour

Even more striking is the fact that one main thesis which has been sponsored by your chairman and others is that we have a sequence of cholesterol progesterone androgens and oestrogens and now according to Dr Huseby that does not necessarily seem to hold He says that there is a good yield of phenolic material without the presence of any detectable progesterone 17 hydroxyprogesterone or androgens I think this is of great importance

Huseby As the chairman knows only too well there is the possibility of many slips between two points and we have not yet identified these phenolic compounds It may well be that since oestrogens are biologically so active there is a small but adequate amount of 17 hydroxylase present in this tumour to produce the oestrogenically active compounds

oestrogen Most, if not all tumours acquire an independence of oestrogen, sometimes by the time they attained transplantable sizes but in other instances only after several transplant generations The interval between initiation and attainment of autonomy may differ greatly in mice of different strains

The intrinsic capacity to become tumours seems to be located in the testis rather than in the other hormone producing or destroying organs of the mice

Some of the triphenylethylene derivatives seem unusually effective in inducing ICT in strains of mice not generally susceptible to them when other oestrogens are given

The tumours produce androgenic effects in the host and occasionally oestrogenic effects They usually have to attain considerable size before the hormonal effects become apparent

REFERENCES

ANDERVONT H B SHIMKIN M B and CANTER H Y (1957) *J nat Cancer Inst* 18 1
ATHIAS M (1945) *Arch Pat Lisbon* 17 397
BISKIND M S and BISKIND G R (1945) *Proc Soc exp Biol N Y* 59 4
BJORN J and GARDNER W U (1956) *Endocrinology* 59 48
BONSER G M and ROBSON J M (1940) *J Path Bact* 51 9
BONSER G M (1942) *J Path Bact* 54 149
BURROWS H (1935) *J Path Bact* 41 218
BURROWS H (1937) *J Path Bact* 44 699
ELY C A (1953) *Proc Soc exp Biol N Y* 84 501
EMMENS C W (1942) *J Endocrin* 3 168
GARDNER W U (1937) *Anat Rec* 68 339
GARDNER W U (1941) *Cancer Res* 1 345
GARDNER W U (1943a) *Cancer Res* 3 92
GARDNER W U (1943b) *Cancer Res* 3 757
GARDNER W U (1945) *Cancer Res* 5 497
GARDNER W U (1954) *J nat Cancer Inst* 15 693
GARDNER W U (1957) Proc 2nd Canad Cancer Conf p 207 New York Academic Press Inc
GARDNER W U and BODDAERT J (1950) *Arch Path (Lab Med)* 50 750
GARDNER W U and STRONG L C (1940) *Yale J Biol Med* 12 543
HOOKER C W PFEIFFER C A and GARDNER W U (1940) *J Amer med Ass* 115 443
HOOKER C W and PFEIFFER C A (1942) *Cancer Res* 2 759

mice with large doses of androgen results in very significant adrenal atrophy

Leathem Is there any relation between oestrogenization and tumour formation in species other than mice? Your strains of mice had varying susceptibilities Prof Gardner And secondly is there any relationship between tumour formation and sensitivity to oestrogen? It seems to me that the mouse testis particularly is less sensitive to oestrogen depression than the rat or guinea pig Giving as much as 25 µg of oestradiol per day we could barely depress the mouse testis and this dose will completely sterilize the adult rat

Gardner We have given oestrogen to dogs for periods of up to 4½ years beginning when they were quite young They retained a very immature type of testis consisting almost entirely of Sertoli cells and spermatogonia No interstitial cell tumours appeared although they are not uncommon in old untreated dogs The amounts of oestrogen were large but much below the amounts that are lethal The horse is interesting because the stallion is one of the best sources of oestrogen There might be some relationship there between seminal resistance to oestrogen and tumour susceptibility In general the strains in which testicular interstitial cell tumours in our experience do occur (the A JK or C strains) are those that are least susceptible to decrease in size after oestrogen treatment Whether one can use decrease in size as evidence of sensitivity or not might merit discussion Mice of the C3H and the CBA strains in which the testes very quickly become very small and quite depleted of interstitial and tubular tissue develop few testicular tumours

Tata Prof Gardner what are anti gonadotrophins? I was wondering if they were similar to the anti pituitary hormones described by Sonenberg and Money where acetylation changes the groupings in the pituitary hormone preparations

Gardner No they are substances that have been prepared by injecting comparatively pure gonadotrophic extracts into rabbits or other animals for quite a long time When the sera from such animals are injected together with known amounts of gonadotrophin they will inhibit the end organ response of the animal They are probably not directly associated with the gonadotrophin because in general the purer the gonadotrophic preparation the less antigenic it is But they are nevertheless quite effective antigenically in a comparatively unpurified state

Pincus What happens to the testes if you use less than total effective doses Dr Huseby? If you use minimal doses and get tubular sclerosis can you get these testes to recover or is it possible to take a cryptorchid testis and replace it in the scrotum?

Huseby We have not carried out experiments in which the testes have been returned to the scrotum I am sure that this would have to be done before extensive tubular sclerosis or calcification had occurred if you were to get anywhere near complete recovery of the tubular elements A point which has been of great interest to us has been that in animals that are just made cryptorchid or that are made cryptorchid and placed

The major part of the phenolic compounds that we see in these incubates may not be biologically active or produced by 17 hydroxylation There is still much work to be done

Dorfman I am ready to support you in part on an alternative pathway for the biosynthesis of oestrogens This fits in very nicely with recent work on the biosynthesis of oestrogens in the pregnant mare a project that was undertaken jointly between our laboratory and that of Dr Heard It revolves around the question of ring B unsaturated oestrogens equilin and equilenin These compounds which are formed in the pregnant mare simultaneously with oestrone are differently labelled The relative specific activity is roughly two to one with oestrone being more highly labelled Furthermore oestrone is not converted to the ring B unsaturated oestrogens so that they do not seem to be simply related There is no direct evidence to indicate that the individual carbon atoms of the molecule have the same specific activity but if we assume this there is an excellent possibility that there exists a second mechanism whereby a biosynthetic pathway to oestrone may include equilin and equilenin which come in part from an endogenous particle (from isoprenoid units) and in part from an exogenous particle The main point is that if this hypothesis is correct oestrogens need not necessarily be formed from androgens (Dorfman R I (1956) *Amer J Med* 21 679)

Furth Your demonstration of $C_{(21)}$ hydroxylase may explain the puzzling behaviour of a transplantable Leydig cell tumour studied extensively by us (Clifton *et al* (1956) *Arch Path (Lab Med)* 62 354) It caused progestational changes in tumour bearing females and a profound atrophy of the adrenal cortex (If the hormonal level was low there was very slight atrophy which could be easily missed but at the beginning of the period there was nearly complete atrophy of the adrenal cortex) Was excessive progesterone overloading the adrenal or is the Leydig tumour itself capable of 21 hydroxylation?

There are a few other puzzling secondary changes not yet mentioned One is the cavernous congestion of the adrenal cortex which goes with hypervolaemia Another is spontaneous rupture of the auricle with exsanguinating haemorrhage also encountered rarely in normal males of the same strain This occurred frequently in animals with grafted highly functional androgenic tumours In general changes indicative of oestrogen production are characteristic of granulosa cells those indicating androgen production of luteomas and Leydig cells

Huseby I am afraid that the detailed analysis of the histology of these tumours in all situations has not yet been completed These tumours are composed mainly of large cells There are however differences in cell morphology in the same tumour line growing in different hosts In other words the histological picture is influenced by whether they have grown in intact females oestrogenized or LH treated males We really are not ready to present the complete story on this however I would like to mention that we have noted adrenal atrophy in animals bearing certain of these tumours but apparently we have not been sufficiently impressed by this since we know that treatment of either male or female

appears to be an increase of the erythrocyte mass (polycythaemic hyper volaemia) In contrast oestrogenic tumours raise the plasma volume (oligocythaemic hypervolaemia with marked relative anaemia)

Gardner These observations might indicate some rather interesting aspects of the cells of origin of these tumours—they might be very primitive or embryonic type cells The origin of the interstitial cells has been much discussed

Yoffey The appearance of erythropoietic foci in the interstitial cell tumours is very intriguing They may be associated with tumour cells but I think one has to be rather careful in interpretation, because whenever you get calcification and subsequent ossification you are liable to get bone marrow developing That in fact was one of the earliest observations made in ectopic bone formation by Neumann shortly after he described the bone marrow in 1868 and it raised one of the earliest controversies which still persists as to what were the cells that were responsible for that it need not necessarily in the present instance be the interstitial cell tumour On the other hand the genito-urinary tract does seem to have an odd association with erythropoiesis I am not thinking so much of the experiments that Maximow did when he ligated the pedicle of the rabbit kidney Of course it promptly died but then it was revascularized from outside became calcified and then fresh bone marrow formed there It may be that the calcification and ossification there were due to the high phosphatase content of the genito-urinary epithelium What I am thinking of is that earlier in vertebrate evolution the mesonephros is one of the main sites of blood formation I could not help speculating along comparative anatomical lines as to whether an additional complication in some of these tumours might be the reassertion of an embryonic trend I have no proof of it but it is a fact that abnormal ectopic haemopoiesis as we call it does tend to re-establish itself in the adult in situations where it had previously appeared in the embryo—in the liver spleen and lymph nodes—and not elsewhere In the early stages of development the testis has as Prof. Gardner knows a close association with the mesonephros and this may somehow be a reassertion of that connexion

Gardner I was pleased to find that Dr Huseby and Dr Furth had made similar observations with regard to testicular tumours I do not think myelopoiesis is necessarily confined only to the testicular tumours I have seen the same condition occasionally in the ovarian tumours and frequently in adrenocortical tumours

Groen I should like to suggest another explanation for the erythropoiesis sometimes seen in these tumours Could it be that a tumour uses up oxygen very intensely when proliferating rapidly? The few stem cells that are left in the interstitium or round the vessels may be stimulated by this relative oxygen lack to undergo a transformation into the haemoglobin producing cells of the bone marrow After all local oxygen lack is a stimulus that will normally induce the bone marrow towards red hyperplasia as Castle has pointed out in another connexion Whether people go up high in the mountains or whether they are anaemic, the local oxygen lack will induce the stem cell in the bone marrow to develop into

on the low dose of stilboestrol we get extensive calcification of the tubules However in animals that are made cryptorchid and whose ovaries are transplanted into the axillary tissue little or no tubular calcification occurs Since the tubular elements which are those next to the basement membrane are retained throughout the life of these animals it is probable that these would be the best for such a study

Pincus I am interested in the effect of oestrogen that you see in the testis of normal individuals which at least as far as the tubules are concerned resembles what you showed

Huseby With the low dose of stilboestrol alone in the diet there are no tubular changes You have to give a fairly high dose to produce tubular changes As I indicated male mice ingesting a diet containing 0 2 µg of stilboestrol per gram were as fertile as were normal animals on the same diet without the oestrogen

Pincus I have seen human testes in oestrogen treated men with definitely sclerosed tubules and very poor interstitial cells yet there has been on the basis of serial biopsies recovery over a period of several months Would you think that these men are likely to develop interstitial cell tumours?

Huseby I cannot answer that one

Gardner I have seen tubular calcification after injection of alcohol into the testis A number of years ago it was assumed that an injection of materials of this particular type might lead to bone formation by the interstitial cells and that was why the alcohol was injected In our experience with mice of the A and JK strains which are susceptible to interstitial cell tumours tubular calcification following high doses of oestrogen is extremely rare and I see no relationship between it and tumour formation In dogs where testicular interstitial cell tumours are very common calcification is also rare I do not recall ever having seen tubular calcification otherwise I wonder if it might not be characteristic of the BALB strain and independent of the interstitial cell tumour potentiality of this strain

Furth I should like to comment on some additional features of androgenizing tumours the tendency towards calcification and the occurrence of erythrogenic foci and many mast cells throughout the tumours Calcification does occur in a wide variety of tumours but it seems to us that it is especially common in transplantable luteomas and Leydig cell tumours Ossification and even marrow formation may follow Most tumours are devoid of mast cells the Leydig cell tumours and luteomas are rich in them

Gardner Myeloid hyperplasia or metaplasia in the interstitial cell tumours is a very interesting observation I do not know about the time effect but certain tumours will show much myeloid tissue hyperplasia and others show none Some will show almost pure erythropoiesis some will show almost a complete bone marrow Some tumours contained bone but this was rare in our experience

Furth Erythropoietic centres occur in these tumours most commonly without bone formation With luteoma and Leydig cell tumours there

DETERMINATION OF SERUM INSULIN IN PATIENTS WITH ISLET CELL TUMOURS OF THE PANCREAS*

J Groen A F Willebrands H G van der Geld† and R E Bolinger‡

Second Department of Medicine Wilhelmina Gasthuis Amsterdam

Introduction

The insulin activity of blood serum in health and disease can be determined *in vitro* by the rat diaphragm method as described previously (Groen *et al*, 1952 Willebrands and Groen 1954 1956) The method although apparently able to detect gross changes in insulin content such as occur in pathological conditions is still not sufficiently accurate for the study of the smaller fluctuations in plasma insulin activity under physiological circumstances A certain discrepancy between the results obtained by us and by other investigators (Randle 1954 1956 Vallance Owen and Hurlock 1954 Vallance Owen Hurlock and Please 1955 Vallance Owen 1956) is probably caused by technical differences and to these we will return later in this paper A factor which is still under investigation and which may also influence the results is the possible presence or absence of substances inhibiting the utilization of glucose by the diaphragm (Bornstein and Park 1953 Field and Dewitt Stetten Jr 1956 Baird and Bornstein 1957 Vallance Owen and Lukens 1957)

The normal values found by us for the insulin content of fasting human serum vary between wide limits viz 0 1 3 0 milliunits per ml

* *These investigations were supported by grants from the Netherlands Society of Diabetic Patients and the Organon Laboratories Inc*

† Holding a fellowship of the American Diabetic Association

‡ Fulbright Research Fellow

the red rather than into the white series Could it be that the same mechanism operates on stem cells lying in close proximity to these tumours? This phenomenon in a spontaneous liver tumour in sheep has been described by Freubel In his preparations you very often see the foci of red cell hyperplasia lying in a niche surrounded as it were by the tumour cells It is as if there was a nest of red cell formation in the centre of the tumour cells where the oxygen tension is so reduced that it stimulates erythropoiesis We have seen the same phenomenon in the gross nodular hyperplasia of the adrenal cortex in our old dogs with spontaneous diabetes

Gardner I never thought of that in particular and I cannot comment on it I did think that androgens might augment erythropoiesis and possibly myelopoiesis That might be due to a production of androgens acting locally in the tumours but we have no proof of this idea

Furth We too have related this effect of increase of erythrocyte mass in general to androgenic hormones and not to anoxia No only are there erythrogenic centres in androgenic tumours but there is also an accompanying enlargement of the spleen and of the liver due in part to formation of erythropoietic centres Androgens could conceivably induce anoxia at the capillary level but this assumption appears far fetched

Yoffey There are many now who would dispute the local action of oxygen deficiency in stimulating erythropoiesis The trend is rather towards the view that the action of oxygen deficiency is indirect evoking the formation of erythropoietin which reaches the haemopoietic tissues through the blood stream

These findings show that the determination of serum insulin may be of value in the diagnosis of hyperinsulinism It should be mentioned that in two of the operated cases (L and R Fig 1) the insulin content of the serum sample was normal on one occasion whereas a second sample taken at a later time gave an elevated value It cannot be excluded that this discrepancy was due to the limited accuracy of the procedure, but it is also possible that the insulin activity of serum of patients with hyperinsulinism varies from day to day so that it may be necessary to perform determinations on more than one occasion

We were naturally gratified by the possibility of making a contribution to the clinical diagnosis of islet cell tumours by the demonstration of increased insulin content of the serum in many cases But we were disappointed because we did not succeed in doing so in all cases and especially in one case where on two occasions we even found low values for the serum insulin This observation induced us to reconsider what we were actually measuring by this method

There was another reason for this reconsideration Although the method has now been used by other authors and the experiments on which we based the evidence that we are really measuring insulin in the serum (Groen *et al* 1952 Willebrands and Groen 1954) have been confirmed (Randle 1954 1956 Vallance Owen and Hurlock 1954 Vallance Owen Hurlock and Please 1955 Vallance Owen 1956) there still exists the rather surprising fact that the values reported by different authors for the normal insulin content vary widely Vallance Owen and his co workers (Vallance Owen and Hurlock 1954 Vallance Owen Hurlock and Please 1955 Vallance Owen 1956) found less than 0 1 m u of insulin per ml of plasma Randle (1954 1956) reported values ranging from 10–20 m u per ml we ourselves find serum insulin values of 0 1–3 0 m u per ml while fasting or resting

Although the techniques used by all these authors are basically the same we have since found that the differences in normal values are at least in part due to the different dilution in the determination When undiluted serum is used

Hyperinsulinism

During the last four years we have examined the serum of 28 patients who were suspected of suffering from hyperinsulinism (Fig 1) Eighteen of these patients were operated on and in 16 cases the diagnosis of an islet cell tumour was confirmed

In eight of these cases of adenoma and in one case of a carcinoma of the islets of Langerhans the serum insulin values found before the operation were definitely above the highest

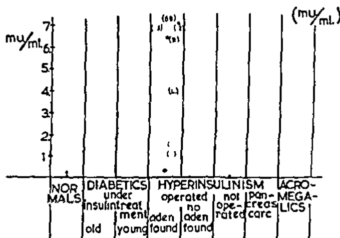


FIG 1 Survey of serum insulin values in health and under different disease conditions

value found for normal serum (3 m u /ml) In fact in three cases the values were extremely high, viz 12, 69 and 73 m u / ml respectively

In two cases operated upon no adenomatous tissue was found in the part of the pancreas that had been removed though the condition of these patients improved after the operation The serum insulin activity in these two cases was increased

The ten remaining patients have not been operated upon so far Six of them are children Four of these ten patients showed elevated serum insulin values

isolated muscle are of the same order as occur physiologically in the blood The concentration of adrenaline in the media in the experiments of Walaas and Walaas (1950) was 1 0 µg /ml while Riesser (1947) used concentrations of adrenaline in the range of 5–10 µg /ml In the literature some disagreement exists as to the physiological level of adrenaline in the plasma Weil Malherbe and Bone (1952) described pressor amine levels of about 5 µg per litre while Valk and Price (1956) using other methods of determination find the average level about 1 µg per litre or less Thus from the previous work on the effect of adrenaline on the carbohydrate metabolism of the rat diaphragm it cannot be decided whether this effect is important at physiological levels since the adrenaline concentrations used were in great excess of the expected physiological values Thus it seemed important to study the effects of various concentrations of adrenaline and of noradrenaline on the effect of insulin on the glucose consumption of the diaphragm in order to arrive at the values for the minimum effective concentrations Furthermore the effect of added adrenaline on the insulin activity of plasma had to be studied

Methods

Rat hemidiaphragms were prepared and placed in flasks according to the method for insulin assay described recently (Willebrands van der Geld and Groen 1958) The insulin concentration in the flasks was adjusted to 0 5 m u per ml Just prior to the incubation after the hemidiaphragms had been put in the media to one flask of each pair a drop (0 03 ml) of adrenaline or noradrenaline solution was added from a calibrated burette to give the desired concentration The adrenaline and noradrenaline used were commercially available synthetic preparations The L adrenaline base was dissolved in a minimum 0 001 HCl solution and diluted with buffer to the desired concentration Noradrenaline was available as the bitartrate

The incubation was carried out as usual at pH 7 4 with shaking at 37° for 90 minutes The glucose concentration of

as incubation medium for the diaphragm tissue, the serum insulin values obtained are considerably less than those found with serum diluted five to ten times with buffer As a possible explanation for this phenomenon it was suggested that serum might contain an inhibiting substance (Bornstein and Park, 1953 Field and Dewitt Stetten Jr, 1956, Baird and Bornstein 1957 Vallance Owen and Lukens, 1957) and we asked ourselves whether the effect of this substance could diminish more rapidly on dilution than that of the serum insulin

Whereas most investigators have been interested in the nature of these possible inhibitory substances as present in patients with severe diabetes, our results in hypoglycaemic states made it more necessary to study the role which the pressor amines might play in this respect Theoretically, adrenaline might complicate the assay procedure either as a secretion of the rat, and thereby present in the diaphragm or as a substance present in the serum to be tested Adrenaline has been known as a hyperglycaemic substance since the work of Cannon (1924), and this effect has been ascribed to an increased release of glucose by the liver and decreased utilization of glucose by the muscles Walaas and Walaas (1950) noted that adrenaline caused a decrease in the glucose consumption of the isolated rat diaphragm and that this was attended by a reduction in the muscle glycogen and an increase in the lactic acid Riesser (1947) showed that the effect of insulin in producing an increase in muscle glycogen could be completely blocked by adrenaline in the media, and Sutherland and Cori (1951) have demonstrated that adrenaline has an effect on phosphorylase such that the inactive form is converted to the active form

That adrenaline exerts an effect on the carbohydrate metabolism of the diaphragm muscle seems therefore to be an established fact, but whether it can play a significant role in the use of the diaphragm muscle as an assay procedure for small quantities of insulin has not been established It would first be necessary to investigate whether the concentrations of adrenaline which influence carbohydrate metabolism in the

on the glucose consumption in concentrations from 0 85 μg /ml to 15 μg /ml A striking effect was noted when adrenaline was added to the media containing insulin in so far as the expected increase in glucose consumption was suppressed in varying degrees dependent upon the adrenaline concentration (Fig 2) Complete inhibition of insulin effect was noted at concentrations of 10^{-2} μg /ml of adrenaline or more and a logarithmic plot of depression at lower concentrations of adrenaline revealed that there was still significant inhibitory effect at concentrations as low as 10^{-4} μg /ml of adrenaline

Noradrenaline A slight but significant inhibitory effect was produced on the basal glucose consumption in the absence of added insulin by noradrenaline in concentrations of 1 2 and 12 μg /ml Again a most striking effect was noted when insulin was present in the medium in a concentration of 5×10^{-4} units/ml Here a depression of insulin effect is complete at concentrations of noradrenaline of 1 μg /ml while significant depression is still noted at concentrations as low as 5×10^{-2} μg /ml (Fig 2) The curve for noradrenaline inhibition is similar in form to that for adrenaline inhibition but shifted towards the higher concentration range so that about 100 times as much noradrenaline is required to give the same inhibitory effect as adrenaline

Effect of adrenaline and noradrenaline on serum insulin activity Upon addition of varying concentrations of adrenaline to serum a suppression of the insulin activity of the ten times diluted serum was noted Fig 3 shows the location of points obtained in this way an approximate agreement occurs with the concentration action curve obtained with pure solutions of insulin and adrenaline It should be added that the serum tested showed in most cases only a rather low insulin activity

Using a serum with higher insulin activity ($2 8 \times 10^{-3}$ units/ml) the inhibition produced by varying amounts of noradrenaline agreed well with the curve obtained for pure solutions of noradrenaline and insulin

The inhibitory effect of adrenaline and noradrenaline upon

the media was determined by the method of Hagedorn Halstrom and Jensen (1946) The apparent insulin concentration in the flask containing insulin plus the pressor amine was calculated as described (Willebrands, van der Geld and Groen 1958), the percentage depression of insulin activity was calculated from the insulin concentration found compared with the expected value of insulin In the experiments involving serum, the serum, diluted ten times with buffer, was substituted for the buffer solution as the medium The glucose concentration in the media was always adjusted to 150 ± 5 mg per cent

Results

Adrenaline The addition of adrenaline to the medium in the absence of added insulin produced only a slight effect

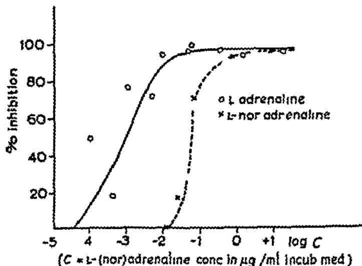


FIG 2 Curve showing relation between inhibition of activity of pure insulin (as measured in the isolated rat diaphragm test) and the concentration of adrenaline or noradrenaline in the medium

On the horizontal on a logarithmical scale concentration of (nor)adrenaline in μg /ml of incubation medium On the ordinate inhibition of insulin activity expressed as percentages of insulin added to the medium

Insulin concentration in all media 0 5 m u /ml

on the glucose consumption in concentrations from 0 85 μg/ml to 15 μg/ml A striking effect was noted when adrenaline was added to the media containing insulin in so far as the expected increase in glucose consumption was suppressed in varying degrees dependent upon the adrenaline concentration (Fig 2) Complete inhibition of insulin effect was noted at concentrations of 10^{-2} μg/ml of adrenaline or more and a logarithmic plot of depression at lower concentrations of adrenaline revealed that there was still significant inhibitory effect at concentrations as low as 10^{-4} μg/ml of adrenaline

Noradrenaline A slight but significant inhibitory effect was produced on the basal glucose consumption in the absence of added insulin by noradrenaline in concentrations of 1 2 and 12 μg/ml Again a most striking effect was noted when insulin was present in the medium in a concentration of 5×10^{-4} units/ml Here a depression of insulin effect is complete at concentrations of noradrenaline of 1 μg/ml while significant depression is still noted at concentrations as low as $5 \times 10^{-}$ μg/ml (Fig 2) The curve for noradrenaline inhibition is similar in form to that for adrenaline inhibition but shifted towards the higher concentration range so that about 100 times as much noradrenaline is required to give the same inhibitory effect as adrenaline

Effect of adrenaline and noradrenaline on serum insulin activity Upon addition of varying concentrations of adrenaline to serum a suppression of the insulin activity of the ten times diluted serum was noted Fig 3 shows the location of points obtained in this way an approximate agreement occurs with the concentration action curve obtained with pure solutions of insulin and adrenaline It should be added that the serum tested showed in most cases only a rather low insulin activity

Using a serum with higher insulin activity ($2\ 8 \times 10^{-3}$ units/ml) the inhibition produced by varying amounts of noradrenaline agreed well with the curve obtained for pure solutions of noradrenaline and insulin

The inhibitory effect of adrenaline and noradrenaline upon

the effect of insulin in increasing the glucose consumption of the diaphragm was noted in our experiments in concentrations much lower than those reported by Walaas and Walaas (1950) and by Riesser (1947) If the physiological blood level for adrenaline is assumed to be between 0 001 and 0 005 μg /ml (Weil Malherbe and Bone 1952, Valk and Price 1956) these levels could be expected to produce an effect on the glucose

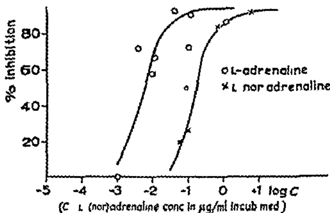


FIG 3 Curve showing relation between inhibition of insulin activity of serum ten times diluted (as measured in the isolated rat diaphragm test) and the concentration of adrenaline or or noradrenaline in the medium On the horizontal on a logarithmical scale concentration of (nor)adrenaline in μg /ml of incubation medium On the ordinate inhibition of insulin activity expressed as percentages of insulin activity added to the medium

consumption of the diaphragm especially when undiluted serum is used for the determinations Willebrands van der Geld and Groen (1958) have noted that higher values for plasma insulin activity are obtained from serum diluted ten times than from whole serum It is therefore quite possible that the dilution of plasma adrenaline that occurs when diluted serum is being used for the determinations would account for the non linearity of the insulin effect on serum dilution For example if a plasma adrenaline level of 10^{-2} μg /ml is present with a given insulin activity, and the plasma is diluted ten

times, the adrenaline level in the media becomes 10^{-3} µg /ml From the curve (Fig 2) however, this dilution of the adrenaline could be expected to produce a change in the inhibitory effect from about 90 per cent to about 50 per cent Assuming that the percentage of depression is independent of the insulin concentration in the range to be expected (10^{-4} — 10^{-3} units/ml) this might result in a serum insulin value calculated from the diluted serum that is five times the value calculated from the undiluted serum Actually this type of relationship was demonstrated in a model dilution experiment to be published elsewhere (Groen *et al* 1958)

The above considerations may be of clinical importance particularly in those cases of insulinoma where even while the blood sugar was quite low normal or even low plasma insulin levels were found Such patients with low blood sugars could be expected to have high adrenaline levels (Valk and Price, 1956 von Euler and Luft 1952 Holzbauer and Vogt 1954) and these might interfere with the results of plasma insulin determinations even where diluted serum is used in the rat diaphragm method

The anti insulin effect of noradrenaline was surprising but is probably not significant at physiological levels

The endogenous adrenaline levels of the test animal must also be reckoned with though at the present time they cannot be controlled in this assay technique It is quite probable that the presence of variable amounts of adrenaline in the diaphragms may account for some of the variation between animals

Finally the fact that the insulin like activity of the plasma is suppressed in a manner similar to that of pure insulin by adrenaline and noradrenaline is further evidence in favour of the fact that this plasma insulin activity in the rat diaphragm test is actually due to insulin

Summary

The determination of serum insulin by the rat diaphragm method can be of help in the diagnosis of islet cell tumours of

the pancreas, but an exact evaluation of the results requires further study, especially of the rôle of adrenaline and possibly of other inhibitors

REFERENCES

BAIRD C W and BORNSTEIN J (1957) *Lancet* 1 1111
BORNSTEIN J and PARK C R (1953) *J biol Chem* 205 503 513
CANNON W B (1924) *Amer J Physiol* 69 46
EULER U S VON and LUFT R (1952) *Metabolism* 1 528
FIELD J B and STETTEN D JR (1956) *Diabetes* 5 391
GROEN J GELD H VAN DER BOLINGER R E and WILLEBRANDS A F (1958) *Diabetes* In press
GROEN J KAMMINGA C E WILLEBRANDS A F and BLEEKMAN J R (1952) *J clin Invest* 31 97
HAGEDORN H C HALSTRØM F and JENSEN B N (1946) *Rep Steno Hosp Copenh* 1 29
HOLZBAUER J and VOGT M (1954) *Brit J Pharmacol* 9 249
RANDLE P J (1954) *Brit med J* 1 1237
RANDLE P J (1956) *J Endocrin* 14 82
RIESSER O (1947) *Biochim biophys Acta* 1 208
SUTHERLAND E W and CORI C F (1951) *J biol Chem* 188 531
VALK A DE T and PRICE H L (1956) *J clin Invest* 35 837
VALLANCE OWEN J (1956) *Diabetes* 5 248
VALLANCE OWEN J and HURLOCK B (1954) *Lancet* 1 68
VALLANCE OWEN J HURLOCK B and PLEASE N W (1955) *Lancet* 2 583
VALLANCE OWEN J and LUKENS F D W (1957) *Endocrinology* 60 625
WALAAS O and WALAAS E (1950) *J biol Chem* 187 769
WEIL MALHERBE H and BONE A D (1952) *Biochem J* 51 311
WILLEBRANDS A F GELD H VAN DER and GROEN J (1958) *Diabetes* In press
WILLEBRANDS A F and GROEN J (1954) *Advanc internal Med* 6 331
WILLEBRANDS A F and GROEN J (1956) *Diabetes* 5, 378

DISCUSSION

von Euler It might help to try serum from adrenalectomized patients since in this case we know at least that the excretion of adrenaline is practically completely suppressed Dr Luft and I have recently made a study of Addisonian patients and we found that in them insulin did not cause any increase in the adrenaline excretion in urine and presumably they do not react to give any increase in the serum either

Pincus Would it be possible to separate adrenaline from insulin without too much difficulty with a simple extraction procedure?

von Euler Insulin might be damaged by for instance treatment with oxygen and say manganous superoxide which would destroy the adrenaline

Pincus Would the adrenaline dialyse out?

von Euler That process is quite slow We have done some experiments by adding adrenaline to serum but the procedure is very slow and it may not be possible to tell whether the insulin survives

Groen We have tried to see whether the insulin activity of blood plasma would dialyse or pass into an ultrafiltrate The insulin activity is not present in dialysates or ultrafiltrates but we have not yet tested whether the inhibitor is there Dr Vallance Owen has tried to detect inhibiting substances in serum dialysates but he has not found them However we are not at all sure if the adrenaline which is present in these very small amounts in the plasma could be detected even if it dialysed out Prof von Euler s suggestion about trying the serum of the adrenalectomized patient is quite a good one because I would suspect that if there is no adrenaline there the determination of insulin in undiluted and in diluted plasma must give the same result

Pincus But there is some noradrenaline present?

Groen It is much less active on the rat diaphragm

Vallance Owen We have not actually studied normal plasma in relation to this dialysis We were particularly interested in diabetes and we studied uncontrolled insulin requiring diabetic patients and depancreatized cats the plasma from which exhibited antagonism to insulin added *in vitro* In neither situation did the antagonism pass through an ultrafilter or through a dialysing membrane So there it appears to be a protein or protein bound substance or possibly a high molecular weight polysaccharide

Pincus As far as the corticosteroids are concerned we might have a steroid protein This is very speculative but it is a possibility that should not be overlooked in view of all the recent work on the protein binding of the steroids

Luft Prof Groen I was somewhat surprised to see that you obtained an increase in blood insulin on acute glucose administration The implication of this would be that an acute increase in the glucose level in blood is a physiological stimulator of the insulin secretion I should like to refer to some earlier studies on the glucose tolerance curve After injection of glucose intravenously the fall of the blood glucose after a time interval of about 20 minutes is linear and the k value i e the disappearance rate of glucose from the blood can be calculated If the test is repeated immediately after the initial one the same k value is obtained Had the first test induced and increased secretion of insulin you would expect to get an increased k value for the second one On the other hand if you perform the second intravenous glucose tolerance test at the end of a two hour intravenous infusion of glucose the k value of this test is lower demonstrating an *increase in plasma insulin* It therefore seems that an acute increase in blood glucose can be handled by the body without an extra amount of insulin while a prolonged elevation of the blood sugar stimulates insulin production These findings

are consistent with the view of Soskin and Levine on the significance of the liver for blood sugar homeostasis

I would also like to comment on the findings of increased insulin levels in your patients with insuloma of the pancreas From your values one might expect to find from five to 20 units of insulin in a total extracellular fluid volume of about 10 litres It is then surprising that intravenous administration of as little as three or four units of insulin to insuloma patients in the fasting state so often induces a hypoglycaemic coma I would like to hear your opinion on this matter

Finally some words about blood insulin in diabetes mellitus Juvenile diabetes is considered as the insulin deficient type of the disease It may be of some interest to note that two such patients after hypophysectomy were able to lead a fairly normal life without any insulin in spite of taking 20–25 mg of cortisone per day One of these patients belonged to our own group of hypophysectomized diabetics the second one was operated on by the group in Gothenburg

Groen We more or less purposely did not do much work on the physiology of insulin as we felt that the method did not justify this perhaps we were too dogmatic there but we would like to try and make the method more accurate first

I have never dared to inject insulin into a patient who was already suffering from hyperinsulinism I have no experience in that way at all

Vallance Owen I have seen it done at the Postgraduate Medical School Glucose is kept ready to administer to the patients as they certainly may go rapidly into coma or at least have a considerable fall in blood sugar

Dorfman What dose would precipitate a coma for these patients?

Vallance Owen To a normal individual 0 1 unit/kg body weight is the usual intravenous dose of insulin whereas 0 05 unit/kg is frequently given to a patient with an insulinoma

As to removing the hypophyses in the experiments carried out in America with Dr Lukens no measurable insulin activity was found in the plasma from depancreatized cats and there was an inhibition of insulin added *in vitro* This inhibition could be removed either by hypophysectomy or bilateral adrenalectomy In either Houssay or Long Lukens cats there was again no plasma insulin activity but now the activity of added insulin was not diminished some 100 per cent being recovered If we injected cortisone or cortisol 10 mg per day for four days into the Long Lukens animal we restored the inhibiting proportion of the plasma The same dose of cortisol injected into Houssay cats for four days and even longer until severe ketosis was produced did not restore the inhibitory activity to the plasma from these animals So it seems at least from these experiments that both the pituitary and the adrenal oxysteroids must be present for insulin antagonism to be found in the plasma of depancreatized cats

It really does not surprise us therefore that if a total hypophysectomy has been performed on your patients Dr Luft insulin antagonism is no longer present in their plasma in spite of the subsequent cortisone injections

Tata Could adrenaline or noradrenaline be bound to proteins? I suggest it would be worth while trying to see what the effect of heavy metal ions on protein bound amines would be We had the problem of getting rid of amino acids phenolic compounds and amines bound to protein we found that a couple of precipitations with zinc and dialysis got rid of all the small molecules which otherwise would come down with the proteins

Pincus That is an excellent point because at the Protein Foundation in Boston Dr Antoniades has actually isolated adrenaline-binding protein and it appears to be moderately stable so dialysis may not be the answer On the other hand was there anything in your dialysate which facilitated the penetration of glucose into the diaphragm Dr Vallance Owen? Was there any insulin like material?

Vallance Owen No we got essentially no effect but this was in the depancreatized cat

Gray What are the fiducial limits of error in your assay Prof Groen?

Willebrands The limits of error are about one third and three this means that when a value of let us say 3 milliunits per ml is found the limits of error are 1 and 9 m u /ml Therefore four of the values found in the patients suspected of suffering from hyperinsulinism were definitely above the upper confidence limit of the highest normal value

Gray These confidence limits are very much better than I have been able to get

Groen As far as your point is concerned Dr Pincus we have lately made fractionation studies with column electrophoresis We found that when we put serum through the column we got endogenous insulin activity not in the albumin fraction but mainly in the β globulin and a little in the γ globulin fraction If we add insulin to the serum this appears in the same fractions none in the albumin most in the β and a little in the γ globulin The same result was obtained when we added radioactive iodine labelled insulin This confirms the impression therefore and all the other evidence we have also points to the fact that the insulin like activity of human serum is indeed due to insulin

Pincus Did you recover all your biological activity in these fractions or was there some you could not find?

Groen We lost some

Willebrands In most of the tested cases the recovery was between the confidence limits of the value to be expected at one time it was less but that was at an unfavourable pH (8 0–8 3) during the electrophoresis

Pincus Were these ion exchange columns?

Willebrands No starch columns

Tata How would insulin alone not added to serum behave on electrophoresis? How would it migrate compared to other serum proteins?

Willebrands The electrophoretic mobility on paper is about the same as α_1 globulin but you have to add a lot of insulin otherwise you find binding of insulin to the paper at the site of application

ADRENAL MEDULLARY AND OTHER CHROMAFFINE CELL TUMOURS

U S von Euler

Karolinska Institute Stockholm

Although chromaffine cell tumours may theoretically develop in all sites where such cells occur they are generally confined to the adrenal medulla or the chromaffine tissue along the abdominal aorta In some cases multiple phaeochrome tumours have been observed mostly of a malignant character Whether these tumours which are often localized in the chest are true metastases or represent a general tendency to tumour growth from scattered normal chromaffine cells is not known

Like the normal chromaffine cells the corresponding tumour cells which have a similar structure produce the two catechol hormones adrenaline and noradrenaline Since the normal adrenal medulla contains separate adrenaline and noradrenaline forming cells (Hillarp and Hokfelt 1953) it might be expected that the tumours would synthesize either one type or the other or a mixture of both While pure nor adrenaline tumours and mixed noradrenaline adrenaline types have been frequently described no case of a pure adrenaline producing tumour however has so far been encountered

Catechol amine content of chromaffine cell tumours

Extracts of chromaffine cell tumours may be assayed bio logically, colorimetrically or fluorometrically all methods allowing differential estimation of noradrenaline and adren aline in a mixture By paper or column chromatography the two catechol amines may be separated and quantitatively estimated

The storage capacity of the tumour chromaffine cell is high like that of the normal chromaffine cell and tumour issue may

contain up to 8 4 mg noradrenaline per g In some tumours the content is very low however and catechol amines may even be absent The adrenaline content is regularly lower and in our material did not exceed 2 3 mg per g No data seem to be available as to the presence of other catechol compounds or their derivatives in chromaffine cell tumours The catechol amine content of the adrenal medullary tumours induced by prolonged treatment with growth hormone in the rat does not seem to have been determined (Moon *et al* 1956)

In 15 cases of phaeochromocytoma in man the following distribution figures were obtained (Table I Euler and Strom 1957)

Table I

ADRENALINE AND NORADRENALINE CONTENT OF CHROMAFFINE CELL TUMOURS

μg/g	*Adrenaline number of cases*	*Noradrenaline number of cases*
0–1	12	3
1–5	3	9
> 5	0	3

Release of catechol amines from tumours

In the majority of cases the catechol hormones seem to be released continuously into the blood from the tumour as is evidenced by the excretion in urine and by the maintenance of clinical symptoms such as increased blood pressure Excretion figures of 8 8 mg noradrenaline per 24 hours have been found corresponding to a production of 220 mg per day assuming an excretion factor of 0 04

The increased excretion of noradrenaline with or without concomitant adrenaline (Engel and Euler 1950) is used as a diagnostic test for the presence of chromaffine cell tumours The excretion figures in 15 cases of phaeochromocytoma are

ADRENAL MEDULLARY AND OTHER CHROMAFFINE CELL TUMOURS

U S von Euler
Karolinska Institute Stockholm

Although chromaffine cell tumours may theoretically develop in all sites where such cells occur they are generally confined to the adrenal medulla or the chromaffine tissue along the abdominal aorta In some cases multiple phaeochrome tumours have been observed, mostly of a malignant character Whether these tumours, which are often localized in the chest, are true metastases or represent a general tendency to tumour growth from scattered normal chromaffine cells is not known

Like the normal chromaffine cells, the corresponding tumour cells, which have a similar structure produce the two catechol hormones, adrenaline and noradrenaline Since the normal adrenal medulla contains separate adrenaline and noradrenaline forming cells (Hillarp and Hokfelt 1953) it might be expected that the tumours would synthesize either one type or the other or a mixture of both While pure nor adrenaline tumours and mixed noradrenaline adrenaline types have been frequently described no case of a pure adrenaline producing tumour, however has so far been encountered

Catechol amine content of chromaffine cell tumours

Extracts of chromaffine cell tumours may be assayed bio logically, colorimetrically or fluorometrically all methods allowing differential estimation of noradrenaline and adren aline in a mixture By paper or column chromatography the two catechol amines may be separated and quantitatively estimated

The storage capacity of the tumour chromaffine cell is high, like that of the normal chromaffine cell and tumour issue may

(Hillarp Lagerstedt and Nilson 1953 Blaschko and Welch 1953)

While the normal chromaffine cell does not release its content spontaneously as is evidenced by studies on the secretion from the denervated gland (Vogt 1952) the tumour cell appears to secrete continuously in many cases This conclusion is based on observations on the urinary excretion in cases of phaeochromocytoma even during complete rest and in the absence of recognized secretion provoking factors

The spontaneous release has been studied in a case of phaeochromocytoma by an analysis of urine collected at intervals of a few hours over a 24 hour period (Bygdeman 1958) An increased output was observed during all periods although the excretion somewhat unexpectedly was highest during the night hours when the patient was asleep increasing from 0 21 μg per minute between 3 p m and 5 p m to 0 61 μg per minute, on an average between 9 p m and 3 a m

During night hours the catechol amine secretion in normal subjects falls to very low figures of the order of 0 01 μg per min Since there is no indication that phaeochrome tumours are innervated it appears that either some circulating factor in the blood or some metabolite in the tumour cell itself causes the release Mechanical factors may also cause a release of hormones a common experience in connexion with surgical manipulation of the tumour

Action of drugs

The release of catechol hormones from chromaffine cells after injection of histamine is the basis for the diagnostic test for phaeochromocytoma introduced by Roth and Kvale (1945) The mechanism for the releasing effect of histamine is unknown but it may be recalled that this amine also increases the response of the homologous postganglionic neurone to preganglionic stimulation (Trendelenburg, 1957) As might be expected *all nicotine like acting compounds release* catechol compounds from the tumour cells Some of these have been suggested for diagnostic purposes

given in Table II, together with the catechol amine content and weight of the extirpated tumour

The maximal output of 8 8 mg noradrenaline during 24 hours was found in a case with multiple unoperable tumours

Table II

ADRENALINE AND NORADRENALINE IN 24 HOUR URINE AND IN EXTIRPATED CHROMAFFINE CELL TUMOUR (EULER AND STRÖM 1957)

Case No	*24 hour excretion in urine*		*Catechol amine mg per g tumour*		*Tumour weight g*
	Adrenaline mg	*Noradrenaline mg*	*Adr*	*Noradr*	
18	<0 01	3 0	0 1	4 0	19
37	<0 01	2 06	<0 1	3 3	11
7	0 02	1 79–2 25	0 1	8 4	50
29	0 01	1 04–1 16	0 25	2 8	85
11	0 02	1 01–1 20	0 07	0 63	40
22	<0 01	0 75–0 80	0 1	1 1	37
19	0 43–0 54	0 45–0 64	1 3	0 63	270
3	0 01–0 05	0 44–1 24	0 1	4 6	45
21	0 01	0 42–1 60	0 1	3 8	17
5	0 16–0 22	0 35–0 38	2 3	7 4	147
13	0 01–0 03	0 24–0 33	0 1	5 2	≃ 4
2	0 03–0 14	0 23–0 88	0 15	1 3	≃ 4
26	< 0 02	0 21–0 62	0 1	1 5	20
1	0 06–0 10	0 13–0 15	1 5	2 1	200 (525)
4	0 11–0 78	0 11–0 66	0 75	0 75	20

As is seen in Table II a close relationship exists between the relative adrenaline content of the tumour and the adrenaline excretion in urine

Of the excreted catechol amines the major portion generally occurs in free form In some cases part of the hormones are excreted in conjugated form By mild acid hydrolysis for 20 min at pH 1 5 an increase to about twice the free amount has been observed

The release mechanism is still largely unknown In all probability the catechol hormones are manufactured and stored in mitochondria like granules as in normal chromaffine cells

The presence of increased amounts of dopamine in urine from patients with chromaffine cell tumours has been shown earlier (Euler 1951 Weil Malherbe 1956)

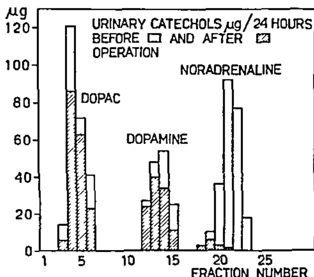


FIG 1 Chromatographic separation of dihydroxyphenyl acetic acid (dopac) dopamine and noradrenaline in extract of urine from a case of phaeochromocytoma before and after surgical removal of the tumour

The excretion figures for dopac dopamine and noradrenaline in urine from normal subjects are given in Table IV for comparison with the figures from the case of phaeochromocytoma already referred to

Recently Armstrong and McMillan (1957) reported excretions of 12 and 90 mg 3 methoxy 4 hydroxymandelic acid per mg creatinine in urine in two cases of phaechromocytoma After operation the excretion figures fell to 1 5 and 2 7 mg respectively It is considered likely that both adrenaline and noradrenaline contribute to the methoxy derivative found in urine

Muscular work

Muscular work is known to increase the adrenaline output from the normally innervated adrenal gland, presumably by increased secretory stimuli through the nerves (Euler and Hellner 1952 Kärki 1956) Work tests performed on a patient with phaeochromocytoma caused a considerable increase in the hormone output (Table III)

Table III

URINARY OUTPUT OF NORADRENALINE IN A CASE OF CHROMAFFINE CELL TUMOUR (EULER *et al* 1955)

Before operation		*After operation*	
Date	*Nor adrenaline in urine µg /min*	*Date*	*Nor adrenaline in urine µg /min*
Sept 18 1954	0 69	Nov 17 1954	0 038
18	0 72	Dec 16	0 028
20		17	0 033
06 00–10 35	0 75	20	
10 35–12 10	1 62	06 00–10 22	0 027
(work test 15 min)		10 22–11 10	0 057
Sept 21 1954	0 72	(work test)	

Operated Sept 30 1954

Excretion of catechol metabolites

A separation of urinary catechols has been obtained from a case of phaeochromocytoma by subjecting the urine extract after adsorption on aluminium oxide and elution to column chromatography and fluorometric estimation of the catechols in the fractions In this way the presence of comparatively large amounts of 3 4 dihydroxyphenylacetic acid (dopac catechol acetic acid homoprotocatechuic acid or HPA) has been demonstrated in addition to dopamine and noradrenaline Fig 1 shows the amounts estimated before and after extirpation of the tumour

strated after lowering the adrenaline content of the gland with insulin (Hokfelt, 1951 Udenfriend *et al*, 1953) Other experiments have indicated a more rapid resynthesis following secretion as a result of splanchnic stimulation (Hökfelt and McLean, 1950, Holland and Schumann, 1956) It appears that resynthesis may proceed at different rates depending on the circumstances The resynthesis of catechol amines in adrenergic nerves occurs at a rapid rate as is evidenced (1) by the continuous release *in vivo* of the neurotransmitter over long periods of time and (2) the fact that no loss in the catechol amine content has been observed in nerves of organs after prolonged stimulation (Luco and Goni, 1948 Euler and Hellner Bjorkman, 1955)

The resynthesis rate in chromaffine cell tumours may be estimated by comparing the urinary excretion in patients with phaeochromocytoma and the actual catechol amine content of the tumour on extirpation The amount of intravenously infused catechol amines which is excreted in urine in normal subjects is 1–4 per cent (Euler and Luft 1951) The figures in Table V are based on the assumption that the urinary excretion is 4 per cent of the catechol amines released into the blood from chromaffine tumours in the material presented by Euler and Strom (1957)

As is seen in Table V the estimated 24 hour release approaches or exceeds the amount present in the tumour in five cases The figures apply to the latest urinary excretion figures obtained before operation It is obvious that the rates of resynthesis represent minimum figures since (1) the release calculated from the urine excretion figures most probably is submaximal (2) it is uncertain whether the whole of the tumour tissue is actively secreting The large variations in the secretion content ratio of the tumours support this assumption

The results thus indicate that the rate of resynthesis in chromaffine cell tumours is relatively high and that the tumour cells are capable of replenishing the stores in less than 24 hours

Table IV

URINARY EXCRETION OF DOPAC DOPAMINE AND NORADRENALINE ESTIMATED FLUOROMETRICALLY AFTER COLUMN FRACTIONATION

	Dopac µg /24 hr	*Dopamine* µg /24 hr	*Noradrenaline* µg /24 hr
Normal subjects (n=5)	79 (32–102)	69 (44–102)	8 9 (7 0–11)
Phaeochromocytoma S J before op	282	146	233
S J after op	204	116	13

The metabolic pathways leading to the synthesis and subsequent inactivation of noradrenaline in cases of phaeochrome cell tumours are represented in Fig 2

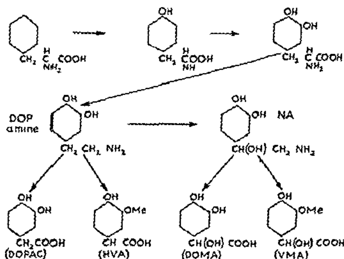


FIG 2 Metabolic pathway of biosynthesis and inactivation of dopamine and noradrenaline (NA)

Resynthesis rate

The rate of resynthesis in the normal adrenal chromaffine tissue is low under certain conditions as has been demon

EULER U S VON (1951) *Ann Surg* 134 929
EULER U S VON GEMZELL C A STROM G and WESTMAN A (1955) *Acta med scand* 153 127
EULER U S VON and HELLNER S (1952) *Acta physiol scand* 26 183
EULER U S VON and HELLNER BJORKMAN S (1955) *Acta physiol scand* 33 Suppl 118 17
EULER U S VON and LUFT R (1951) *Brit J Pharmacol* 6 286
EULER U S VON and STROM G (1957) *Circulation* 15 5
HILLARP N A and HOKFELT B (1953) *Acta physiol scand* 30 55
HILLARP N A LAGERSTEDT S and NILSON B (1953) *Acta physiol scand* 29 251
HOLLAND W C and SCHUMANN H J (1956) *Brit J Pharmacol* 11 449
HOKFELT B (1951) *Acta physiol scand* 25 Suppl 92
HOKFELT B and MCLEAN J (1950) *Acta physiol scand* 21 258
KARKI N T (1956) *Acta physiol scand* 39 Suppl 132
LUCO J V and GONI F (1948) *J Neurophysiol* 11 497
MOON H D KONEFF A A LI C H and SIMPSON M E (1956) *Proc Soc exp Biol N Y* 93 74
ROTH G M and KVALE W F (1945) *Amer J med Sci* 210 653
SJOERDSMA A LEEPER L C and UDENFRIEND S (1957) *Fed Proc* 16 336
TRENDELENBURG U (1957) *J Physiol* 135 66
UDENFRIEND S COOPER J R CLARK C T and BAER J E (1953) *Science* 117 663
UDENFRIEND S and WYNGAARDEN J B (1956) *Biochim biophys Acta* 20 48
WEIL MALHERBE H (1956) *Lancet* 2 282
VOGT M (1952) *Brit J Pharmacol* 7 325

DISCUSSION

Boyland Glucuronidase is present in the urine so if glucuronides were excreted and the urine allowed to stand the glucuronidase present might hydrolyse the noradrenaline glucuronide How quickly after the urine was passed was it examined?

von Euler In several cases we have analysed the urine in special samples within an hour or so

Boyland And you found no difference between the fresh and the hydrolysed?

von Euler We have only done a few hydrolysis experiments since lately we have only studied the free amounts It seems that in normal urine at least the extra amount found on hydrolysis is not very significant The usual increase is about twice the amount and we thought we had better stick to the free since there was apparently a good correlation between free and conjugated

Boyland How much hydroxytyramine is excreted?

von Euler Between 50 and 100 µg each day

Table V

Relationship between calculated output of catechol amines from chromaffine cell tumour (estimated from urinary excretion) and catechol content of tumour (at operation)

Case	*Estimated catechol secretion from tumour per 24 hours*		*Catechol amine content of tumour*		*24 hour excretion in per cent of tumour content*	
	adr mg	*noradr mg*	*adr mg*	*noradr mg*	*adr*	*noradr*
5	5 6	9 6	340	1090	1 6	0 88
19	13 5	16	350	170	3 9	9 4
7	< 1	45	< 2 5	420		11
3		24 2	4 5	207		11 7
9		29	21	248		12
13		8 1	< 1	≃21		38
22		20	3 7	41		49
26		15 4	2 0	30		51
21		40	1 7	65		62
18		75	1 9	76		99
4	19 5	16 5	15	15	130	110
2	< 1	5 9	< 1	≃ 5 2		≃113
11		30	2 8	25		120
37		51 5	< 1	36 3		142

Using radioactive tracer technique Sjoerdsma Leeper and Udenfriend (1957) found a rapid turnover of noradrenaline (half life of 7–9 hours) in a case of phaeochromocytoma, compared with a half life of about 240 hours in the suprarenal gland after depletion with insulin (Udenfriend and Wyngaarden, 1956)

The present material does not suggest any difference in the rates of synthesis of adrenaline and noradrenaline

REFERENCES

Armstrong M D and McMillan A (1957) *Fed Proc* 16 146

Blaschko H and Welch A D (1953) *Arch exp Path Pharmal* 219 17

Bygdeman S (1958) To be published

Engel A and Euler U S von (1950) *Lancet* 2 387

of the animals were dead and two were moribund I removed the tum ours from the four mice and transplanted three of the grafts into three other animals and it killed them That was really a malignant tumour! The second time we transplanted tumour tissue weighing 3–5 mg and it killed every animal we put it into We removed the graft from one animal which was moribund and retransplanted it This host survived but the tumour has not grown Histologically these tumours contained some chromate combining material but we have not done any chemical tests on it These may be identified in the animals by palpation but we never know whether we are going to get a cortical or a medullary tumour until we examine the animal at autopsy

Pincus In giving the excretion values for dihydroxyphenylacetic acid (dopac) and dopamine you pointed out that these were not as good indices particularly in the effect at operation as noradrenaline itself Were those values elevated as compared to normal? Because if they were I suggest that there is a source of precursor presumably outside the tumour

The case where you got a very high excretion during the night is the reverse of the usual diurnal rhythm for adrenaline and noradrenaline as studied in our laboratory If this is a consistent finding does the blood pressure in these patients tend to increase during the night or is that just an accidental event?

von Euler In the case of phaeochromocytoma the excretion of both dopac and dopamine was higher than normal The increase was not in proportion to the noradrenaline and that may be partly due to the fact that not all but only certain proportions of the dopamine were going through the noradrenaline channel so to speak and it just proceeded on its own as being cast aside Therefore it might be a little bit dangerous to rely on the dopamine metabolites only On the other hand Armstrong s suggestion of using methoxy hydroxymandelic acid would be better unless noradrenaline were found

In this case of phaeochromocytoma we were only able to follow it over two 24 hour periods and then the patient had to be operated on I can quite confirm that the excretion in normal subjects is not as high during the day as during the night On an average the noradrenaline excretion was three or four times higher at night in the case of phaeochromocytoma We have no explanation whatever for these increased figures admittedly it was only one case but the same results were obtained on the second night

Dorfman Is it possible that there was some form of medication that escaped your eye?

von Euler These were independent tumours!

Dorfman As far as I know this finding of the methoxy derivative of catechol amine was the first instance of a methoxy compound in animal sources Within a relatively short period of time a methoxy derivative in the oestrogen series has been found by Dr Gallagher and by Dr Lewis Engel Up to that time methoxy derivatives in nature had only been known in plant sources How about methoxy derivatives of the thyroid hormones?

Dorfman What about the stability of the materials in the extraction and purification procedures that you employ?

von Euler We were rather afraid of this at the beginning of the treatment of which the first step is adsorption on aluminium oxide in alkaline solution followed by elution and the whole procedure of column chromatography But the recovery has been fairly good at least of hydroxyphenylacetic acid dopamine adrenaline noradrenaline and *iso*propylnoradrenaline For all of these the average recovery is about 70 per cent—I should say it varies between 60 and 80 per cent

Dorfman So it is possible that 20–30 per cent may be lost Our studies indicate somewhat larger losses For this reason we have worked with the acetyl derivatives which seem to give a recovery closer to 90 per cent

Furth Among our recent lucky findings is a transplantable chromaffine cell tumour in the mouse Paul Hagen assayed it and found adrenaline and noradrenaline in the grafted tumours in amounts only slightly less than those present in the normal medulla What impressed us most when this transplantable adrenal tumour was discovered was that the hosts did not have the usual effects of adrenal tumours There was no gonadal activity the adrenals of the tumour hosts were yellow and of normal size and the thymus was normal The chromaffine matter of the host s adrenal was not decreased This suggests that there is no feedback mechanism such as exists with other endocrine tumours The hosts appeared to be in good health Some homeostatic adjustment must exist in these hosts or else the adrenaline must be bound to the tumours because when tumour tissue was injected intramuscularly most animals promptly died of shock Transplantation is facilitated by the use of small inoculum or sedimented tumour cells Unfortunately the tumour became highly malignant and chromaffinity vanished

von Euler Is there any indication that this tumour does secrete? Has the urine been investigated or analysed? If a considerable amount was excreted in the urine that would indicate a secreting tumour but otherwise the animal could probably bear quite a good sized tumour full of noradrenaline It was also noted by Li and his group when they found the chromaffine cell tumours in rats that there was no increased blood pressure I think it would have been better to measure the output in urine

Furth The urine was not assayed Lack of enlargement of the heart and of arteriosclerotic changes suggest that if hypertension was present it was mild

Gardner We have seen many of these phaeochromocytomas in mice over quite a few years and we first reported them in 1940 Many are quite small although some of them are as large as a kidney So far in our experience we have never seen one in thousands of control animals but in probably 1 500 or 1 600 irradiated or gonadectomized animals we have had 30–40 of them if not more than that So far we have never successfully transplanted them because they killed every animal we put them into The first time I transplanted one I put the tumour into six animals and when I went to the animal laboratory two hours later four

GENERAL DISCUSSION

Pincus The problem of opening the general discussion is one which has led me to suspect the hospitality of my host However I think that I can make a few simple points about our proceedings

In the discussion of these hormone producing tumours it has been obvious I think that there are three classical aspects which some times have not been separated or separable (*a*) the first is concerned with aetiology the factors responsible for the origin of hormone producing tumours (*b*) the second might be designated the problem of pathogenesis or the nature of the tumours once they are developed and (*c*) the third is the problem of specific diagnosis

The most surprising thing to me as I hope an impartial observer has been the striking effect in experimental animals of endocrine imbalances of one sort or another For example there are the very remarkable repercussions of the effect of castration or irradiation leading to endocrine imbalance in the experimental animals and the administration of hormone or the administration of goitrogen All of these procedures have proved very efficacious in causing a great variety of tumour developments The probability of interrelation ship between one glandular system and another has only been touched on here and I think that remains a very interesting and largely unexplored area Prof Gardner for example happened to remark during the last discussion on the occurrence of the chromaf fine cell tumours in his experimental animals Here is a system the adrenal medulla which we have thought to be pretty much exempt from the familiar pituitary adrenocortical axis and yet it is definitely being affected by influences which we thought primarily affected that axis In other words the extensions of the effects of hormonal imbalance appear to be much wider than I personally had imagined before coming to this meeting

Another feature of the discussion which seems to me to require comment is the great diversity of effect once the tumour is established In steroid producing organs we may have almost any conceivable or inconceivable upsets of the steroidogenic process We have taken per haps too much as an article of faith that there is one particular scheme of steroidogenesis I suspect that we are going to have to revise our ideas considerably since it has been shown that exactly the same treatment leads to androgen producing tumours on the one hand oestrogen producing tumours on the other and also to tumours which produce corticosteroid like substances from the testis (as in

Tata There was a report some years ago saying that thyroxine methoxylation takes place in the phenolic group So that would be the product to use for comparison It is very difficult to methoxylate thyroxine chemically

Boyland There are two other examples which were demonstrated quite recently One is that xanthurenic acid is methylated in the body to an 8 methoxykynurenic acid another is the methylation and dehydroxylation of 3 4 dihydroxybenzoic acid

GENERAL DISCUSSION

Pincus The problem of opening the general discussion is one which has led me to suspect the hospitality of my host However I think that I can make a few simple points about our proceedings

In the discussion of these hormone producing tumours it has been obvious I think that there are three classical aspects which sometimes have not been separated or separable (*a*) the first is concerned with aetiology the factors responsible for the origin of hormone-producing tumours (*b*) the second might be designated the problem of pathogenesis or the nature of the tumours once they are developed and (*c*) the third is the problem of specific diagnosis

The most surprising thing to me as I hope an impartial observer has been the striking effect in experimental animals of endocrine imbalances of one sort or another For example there are the very remarkable repercussions of the effect of castration or irradiation leading to endocrine imbalance in the experimental animals and the administration of hormone or the administration of goitrogen All of these procedures have proved very efficacious in causing a great variety of tumour developments The probability of interrelationship between one glandular system and another has only been touched on here and I think that remains a very interesting and largely unexplored area Prof Gardner for example happened to remark during the last discussion on the occurrence of the chromaffine cell tumours in his experimental animals Here is a system the adrenal medulla which we have thought to be pretty much exempt from the familiar pituitary adrenocortical axis and yet it is definitely being affected by influences which we thought primarily affected that axis In other words the extensions of the effects of hormonal imbalance appear to be much wider than I personally had imagined before coming to this meeting

Another feature of the discussion which seems to me to require comment is the great diversity of effect once the tumour is established In steroid producing organs we may have almost any conceivable or inconceivable upsets of the steroidogenic process We have taken perhaps too much as an article of faith that there is one particular scheme of steroidogenesis I suspect that we are going to have to revise our ideas considerably since it has been shown that exactly the same treatment leads to androgen producing tumours on the one hand oestrogen producing tumours on the other and also to tumours which produce corticosteroid like substances from the testis (as in

hypophysectomized gonadectomized adrenalectomized animal can produce a mammary gland which will cause secretion of milk?

Furth Yes with some co factors Our experiments suggest that this is a specific cell whose main function is to stimulate the mammary gland Grafted mammotropes do so in the absence of the hypophyses Oestrogens are the physiological stimulants of mammotropes Autonomous secreting mammotropes cause great enlargement of mammary glands with extensive secretion even in hypophysectomized and gonadectomized animals

In the chronically stimulated gland there are multicentric hyperchromatic areas and adenomas One major problem is that of the relation of this mammary hyperplasia to mammary cancer At first, we underestimated the role of progesterone and corticoids Apparently progesterone reduces secretory and increases proliferating activity Corticoids alone will not produce hyperplasia of the mammary glands but they have a powerful permissive action with respect to the function of the mammotrope The situation calls for systematic quantitative studies with respect to progesterone and corticoids in hypophysectomized animals bearing grafted mammotropes Briefly the fulcrum of mammary gland influences is the mammotrope Oestrogen does not act directly on the mammary gland The action of the mammotropes is modified by a number of factors determining the degree (and kind?) of secretion and growth of the mammary gland

Gardner It has been known since 1933 that oestrogen will not induce growth in the mammary glands of hypophysectomized animals and since 1941 that the lactogenic hormone plus oestrogen would produce growth of the mammary gland in hypophysectomized mice Now this is presumably not the same effect that Dr Furth has shown here where the mammary glands were well developed and lactating In the male rat the mammary glands are almost always well developed To induce lactation is difficult in a hypophysectomized animal I do not think anybody other than Dr Lyons has ever done that with any combination of the pituitary, adrenocortical and gonadal hormones

Leathem Our own efforts at combination have generally failed to produce glands of the secretory state that you have found

Pincus Dr Woolley the suggestion that early ovariectomy leads to adrenal tumorigenesis in animals made me speculate about the human We have cases of ovarian agenesis that are essentially of foetal origin Is there any indication that in such women there is a high incidence of adrenal tumours?

Woolley There has been an attempt to see whether or not nodular hyperplasia of the adrenal cortex is more frequent in women past the

menopause than earlier for example when ovarian function fails and also in females as compared to males I am not sure that the evidence stands up very solidly at the moment but some investigators feel that there is an increase of nodular hyperplasia of the adrenal cortex in postmenopausal women

Pincus Have you any parallel experiences with late ovariectomy in animals?

Woolley Yes up to 15 months in certain strains Ovariectomy does not have to be early but evidence has not been secured that there is more rapid formation at the later ages

Pincus Does that mean that when people are living beyond the century mark they will all die of adrenal tumours?

Dorfman More likely they will be adrenalectomized by then!

Gardner I have often been concerned at the amount of knowledge there is about mice and why so many mice have so many different tumours Is it because the geneticists have helped us to mix things up a bit or is it that these animals live pretty fast? Could there be any relationship between the general metabolic rate per unit of tissue and the tendency for some of these things to happen I think we should ask ourselves why as Dr Pincus has said there are so many tumours in these small animals There are fewer in the rat in the rabbit and possibly in man

Dorfman Maybe we just look at more mice

Woolley It is difficult for many laboratories to maintain guinea pigs and certain other animals to old age to observe tumour incidence due to the length of time involved

Groen Tumours develop very commonly in almost all dogs if they grow old enough They almost all develop something Adenoma of the breast glands in female dogs is very common and in fact very few elderly female dogs do not have one or more of these tumours Prostatic adenomas occur in old male dogs Nodular hyperplasia of the adrenals in female dogs over 11 or 12 years of age is almost universal and I am sure that the veterinary surgeons must know of even more examples It is very interesting that just as better general hygienic conditions and better nutrition have prolonged the average life span of Western man so also his pet animals especially his dogs are better cared for so that the number of old animals is rapidly increasing If the owners would only part with them the study of these spontaneous tumours in old dogs might be just as fruitful as in old mice

Leathem Lymphomas are extremely common in dogs We have a dog cancer clinic and 53 of them with lymphomas were made available to us last year by the veterinarians in the State of New Jersey These dogs were aged anywhere from four or five years and on so that they do also occur at early ages

AUTHOR INDEX TO PAPERS

SUBJECT INDEX

Printed by Spottiswoode Ballantyne & Co Ltd London and Colchester

CUMULATIVE INDEX
TO
VOLUMES 1–12

CUMULATIVE INDEX TO VOLUMES 1–12

Print d by Spottiswood Ballantyne & C Ltd London and Col h te